# Reproduction of European Eel in Aquaculture (REEL)

## Consolidation and new production methods

**DTU Aqua Report 249-2012**

By Jonna Tomkiewicz (ed.)

**DTU Aqua**
National Institute of Aquatic Resources

# Reproduction of European Eel in Aquaculture (REEL)

Consolidation and new production methods

## DTU Aqua Report 249-2012

Editor: Jonna Tomkiewicz

Authors: Jonna Tomkiewicz, Lars Tybjerg, Josianne G. Støttrup, Fintan McEvoy, Peter Ravn, Sune Riis Sørensen, Peter Lauesen, Christian Graver, Peter Munk, Lars K. Holst, Bjarne Vestbö, Eiliv Svalastoga, Charlotte Jacobsen, Bjørn Holst, Svend J. Steenfeldt, Lene Buelund, Thomas Hornum, and Tanja Kofoed

**Denmark and the European Union invest in sustainable fisheries and aquaculture. This project is financially supported by the Danish Ministry of Food, Agriculture and Fisheries and the European Commission**

Ministry of Food, Agriculture and Fisheries

The European Fisheries Fund

## Data sheet

**Reproduction of European Eel in Aquaculture (REEL)**
Consolidation and new production methods

Editor: Jonna Tomkiewicz

Authors: Jonna Tomkiewicz, Lars Tybjerg, Josianne G. Støttrup, Fintan McEvoy, Peter Ravn, Sune Riis Sørensen, Peter Lauesen, Christian Graver, Peter Munk, Lars K. Holst, Bjarne Vestbö, Eiliv Svalastoga, Charlotte Jacobsen, Bjørn Holst, Svend J. Steenfeldt, Lene Buelund, Thomas Hornum and Tanja Kofoed

June 2012
National Institute of Aquatic Resources

DTU Aqua Report No 249-2012
ISBN 978-87-7481-154-1
ISSN 1395-8216

Cover Design: Peter Waldorff/Schultz Grafisk
Cover Photo: Peter Jensen

Reference: Tomkiewicz, J. (ed.). Reproduction of European Eel in Aquaculture (REEL): Consolidation and new production methods. DTU Aqua Report No 249-2012. National Institute of Aquatic Resources, Technical University of Denmark, 47 p.

## Preface

This report presents results of the project, Reproduction of European Eel in Aquaculture: Consolidation and new production methods that was conducted during the period 1st October 2008 to 31th December 2010. The project received financial support from the Danish Ministry of Food, Agriculture and Fisheries and the European Commission through the European Fisheries Fund (EFF). The REEL project addressed the EFF initiative "to optimise aquaculture production including reduction in resource utilisation and environmental improvements, enhancement of recirculation technology and farming systems as well as development of a cost effective fry production".

The project applied an interdisciplinary approach integrating knowledge institutes and industry including SMEs in method and technology development. REEL was coordinated by the Technical University of Denmark, National Institute for Aquatic Resources (DTU Aqua) and participants included Copenhagen University, Faculty of Life Sciences (KU-Life), Danish Eel Farmers Association, Billund Aquaculture Service APS, BioMar A/S, and Bioneer A/S. The following persons contributed to this collaborative research and development project:

| | |
|---|---|
| **Technical University of Denmark (DTU)** | *Jonna Tomkiewicz, DTU Aqua* |
| | *Sune Riis Sørensen, DTU Aqua* |
| | *Josianne G. Støttrup, DTU Aqua* |
| | *Charlotte Jacobsen, DTU Food* |
| | *Peter Munk, DTU Aqua* |
| | *Svend J. Steenfeldt, DTU Aqua* |
| | *Lars Tybjerg, DTU Aqua* |
| | *Thomas Hornum, DTU Aqua* |
| | *Tanja Kofoed, DTU Aqua* |
| **Copenhagen University,** | *Eiliv Svalastoga* |
| **Faculty of Life Sciences (KU-Life)** | *Fintan McEvoy* |
| | *Lene Buelund* |
| **Billund Aquaculture Service APS (BA)** | *Peter Lauesen* |
| **Danish Eel Farmers Association (DEFA)** | *Christian Graver* |
| **BioMar A/S(BioMar)** | *Lars K. Holst* |
| | *Bjarne Vestbö* |
| | *Niels Hjermitslev* |
| **Bioneer A/S(Bioneer)** | *Peter Ravn* |
| | *Bjørn Holst* |

Inger Hornum (DTU Aqua), Thi Thu Trang WU (DTU Food) and additional staff from the participants contributed to the results and success of the project. Maria Krüger-Johnsen (DTU Aqua) assisted in the editing of the report.

Charlottenlund, March 2012

Jonna Tomkiewicz, Project Coordinator REEL

# Table of contents

## Summary

# Reproduction of European eel (REEL): Consolidation and new production methods

*Project aim:* Enhance methods and technology applied to produce and culture European eel larvae as basis for the development of a future self-sustained eel aquaculture.

*Background:* The severe decline of the European eel stock calls for conservation measures including national eel management plans and establishment of a self-sustained eel aquaculture. In 2005, the National Institute of Aquatic Resources at the Technical University of Denmark (DTU Aqua), the Faculty of Life Sciences at Copenhagen University (KU-Life) and the eel aquaculture industry started to build up a research and technology platform for the development of methods to reproduce European eel in aquaculture. Two major projects: Artificial Reproduction of Eels II and III (ROE II and III) succeeded during 2005-2008 to produce viable eggs and larvae that lived up to 12 days. The larvae thereby accomplished the yolk-sac stage and became ready to start feeding. The results were in particular promising because they evidenced that methods successfully applied to Japanese eel has a potential for application also to the European eel. ROE II and III were supported by the Ministry of Food, Agriculture and Fisheries and the European Commission through the Financial Instrument for Fisheries Guidance (FIFG) and the Danish Food Research Program 2006, respectively.

*Results:* The REEL project accomplished through three series of experiments to consolidate previous results. The longevity of larvae was extended from 12 to 20 days after hatch in first feeding experiments thereby entering the leptocephalus phase. Maturation potential and methods to induce maturation were further tested, and farmed and wild eel broodstocks as well as different treatments were compared. In particular, fertilisation procedures to produce fertilised eggs and embryos and monitoring techniques were enhanced. The technology needed to culture embryos and larvae was substantially improved. The potential for new hormonal treatments was explored and recombinant eel hormones have been produced. New broodstock diets were developed with focus on the lipid composition essential for development and survival of fish larvae. In addition, the experimental facility established by DTU Aqua at Lyksvad Fish Farm was enhanced by improving the experimental and laboratory facilities.

The REEL project provided the basis for the establishment of an EU collaborative research project: *Reproduction of European Eel: Towards a Self-sustained Aquaculture* (PRO-EEL) coordinated by DTU Aqua. REEL included the partners DTU Aqua, KU-Life, Danish Eel Farmers Association (DEFA), Billund Aquaculture Service (BA), BioMar, and Bioneer of which four are integrated in the PRO-EEL project that in total has 15 international partners.

## Resumé (*in Danish*)

# Reproduktion af europæisk ål (REEL): Konsolidering og nye produktionsmetoder

*Projektets formål:* Udvikling af metoder og teknologi til produktion og kultur af ålelarver som grundlag for udviklingen af et selvforsynende akvakulturerhverv.

*Baggrund:* Den drastiske tilbagegang i bestanden af europæisk ål stiller krav til bevarelses-foranstaltninger herunder nationale forvaltningsplaner for ål og etablering af et selvforsynende åleopdræt. I 2005 begyndte Danmarks Tekniske Universitet, Institut for Akvatiske Ressourcer (DTU Aqua), Københavns Universitet, Det Biovidenskabelige Fakultet (KU-Life) og åleakvakultur-industrien i Danmark at opbygge en forsknings- og teknologiplatform med henblik på udvikling af metoder til at reproducere ål i akvakultur. Det lykkedes i perioden 2005-2008 i to større projekter: *Kunstig Reproduktion af ål II og III (ROE II og III)* at producere levedygtige æg og klække larver, der levede i op til 12 dage. Larverne gennemlevede derved det såkaldte blommesækstadium og blev klar til selv at tage føde til sig. Resultaterne var især lovende, fordi de viste, at metoder, som med succes er anvendt til japanske ål også kan anvendes til den europæiske ål. ROE II og III blev støttet af Ministeriet for Fødevarer, Landbrug og Fiskeri og EU Kommissionen gennem Det Finansielle Instrument til Udvikling af Fiskeriet (FIUF) og Fødevareforskningsprogrammet 2006.

*Resultater:* REEL projektet har gennem tre serier af eksperimenter konsolideret tidligere resultater. Levetiden for larver blev udvidet fra 12 til 20 dage efter klækning i de første larvefodrings-eksperimenter, hvorved larverne gik ind i det såkaldte leptocephalus-stadium. Modningspotentiale og metoder til at inducere modning er blevet yderligere testet, og forskelle mellem opdrættede og vildtlevende moderfisk samt mellem forskellige behandlinger er undersøgt. Især er metoder til befrugtning af æg samt produktion og kvantificering af æg og fostre blevet forbedret. Også den nødvendige teknologi til kultur af befrugtede æg og larver er blevet væsentligt forbedret. Potentialet

for nye hormonbehandlinger er udforsket, og det er lykkedes at producere ålehormoner ved hjælp af rekombinant DNA teknologi. Forbedret foder til moderfisk er udviklet med fokus på fedtsyresammensætningen, som er af afgørende betydning for udviklingen og overlevelsen af marine fiskelarver. Hertil kommer, at den eksperimentelle facilitet etableret af DTU Aqua på Lyksvad Fiskefarm er blevet styrket gennem forbedring af forsøgs- og laboratoriefaciliteter.

REEL projektet har dannet grundlag for etablering af et EU-forskningsprojekt: *Reproduction of European Eel: Towards a Self-sustained Aquaculture* (PRO-EEL) som ledes af DTU Aqua. REEL omfatter partnerne DTU, KU-Life, Dansk Åleproducentforening, Billund Akvakulturservice, BioMar, og Bioneer, hvoraf fire er integreret i PRO-EEL. Projektet omfatter i alt 15 internationale partnere.

# 1 General introduction

The European eel stock has declined drastically over the past 30 years and there are no signs of recovery. As a consequence, the European Commission requested in 2007 national management plans that should be implemented in Europe to protect the diminishing eel stock. This included Denmark, where a management plan for eels has been developed by the Ministry of Food, Agriculture and Fisheries in collaboration with DTU Aqua, Danish Aquaculture Association and the Danish Environmental Agency. The depletion of the eel stocks also urges the need to establish a self-sustained eel aquaculture to replace the present capture based aquaculture that relies on wild caught glass eel. Hence, reproduction of eel in captivity has become an indicated goal of the European Commission and national governments.

Research on reproduction of eel in captivity has a long tradition in DTU Aqua dating back to the work of Inge and Jan Boëtius during the period from 1970ies to 1990ies at the former Danish Institute for Fisheries and Marine Research. The recent research and technology development for reproduction of eel in aquaculture at DTU Aqua started in 2005 in collaboration with the eel aquaculture industry including the Danish Eel Producers Association, Billund Aquaculture Service, BioMar as well as Copenhagen University. The projects received financial support from the Ministry of Food, Agriculture and Fisheries and the European Commission. The two major projects: *Artificial Reproduction of Eels II* and *Reproduction of Eels III (ROE II* and *III)* conducted from 2005-2008 were supported through Financial Instrument for Fisheries Guidance (FIFG) and the Danish Food Research Program 2006, respectively.

Within the ROE II and ROE III projects, the research team succeeded in producing viable eggs and larvae that lived up to 12 days. These larvae developed and completed the yolk-sac stage during which they fully depend on the nutrition from the egg and reached the leptocephalus larval stage. At this stage the larvae are fully developed and ready to start feeding on their own. These results brought Denmark to a leading position within experimental research on reproduction of European eel in culture, breaking the former Russian record of larvae living up to 3.5 days in a mass hatching of larvae. The results were in particular promising because they evidenced that the method successfully applied to Japanese eel has a potential for application also to European eel.

The research and technology development conducted in the present project: *Reproduction of European eel (REEL): Consolidation and new production methods* (2008-2010) represent a further step towards a future production of glass eels for European eel aquaculture. The aim of the REEL project was to consolidate the methods and technologies established in ROE II and III and explore the potential for development of novel hormonal treatments. Similar to ROE II and III the project integrates industry and knowledge institutes extending the consortium with the bio-engineering company: Bioneer.

During the project period, experiments were carried out with the purpose to induce maturation in established farmed and wild eel broodstocks, perform *in vitro* fertilisation, produce viable eggs and yolk-sac larvae, and establish culture conditions for larvae until the onset of feeding. The potential for new hormonal treatments were explored through recombinant production of an eel hormone. New broodstock diets were developed with focus on the lipid composition essential for development and survival of fish larval. The project succeeded in enhancing the quality of

broodstocks, the egg production success and the larval longevity. The present record set by the project is larvae that lived up to 20 days post hatch in a pilot feeding experiment.

The infrastructure applied has been enhanced by improving the experimental and laboratory facilities. This includes establishment of adequate laboratory conditions and extending cold storage opportunities. The recirculation systems for broodstock experiments have been equipped to enable temperature regulating and incubation and larval rearing facilities have been extended. In addition, PLC systems have been integrated for monitoring and steering physical-chemical parameters and control light regime.

The REEL project thereby provided the basis for the establishment of an international collaborative research project: *Reproduction of European Eel: Towards a Self-sustained Aquaculture* (PRO-EEL) funded under the Seventh Framework Programme of the European Commission. The PRO-EEL project coordinated by DTU Aqua, started in April 2010 and ends 2014. It integrates 15 partners from leading research institutes including internationally recognized aquaculture institutes and four of the Danish participants included in the REEL project from the industry and universities i.e. Billund Aquaculture Service, BioMar, Copenhagen University and DTU Aqua. In PRO-EEL, the Danish research facilities and the integrated concept of DTU Aqua form the backbone in the continued efforts to establish a production of glass eels.

# 2 Project objectives, contents and consortium

The aim of the REEL project was to consolidate and further develop methods and technology using protocols and products, methods and technologies established in previous projects as starting point. The purpose of the standardisation of procedures and technology development was to stabilize the production of viable yolk-sac larvae and establish larvae cultures ready for feeding.

## 2.1 Objectives

Research development, monitoring and documentation of production methods, products and technology were addressed in the experimental work. Specific objectives included to:

1. improve egg and semen quality,
2. stabilise production and survival of eggs and larvae,
3. establish suitable incubation and culture conditions for larvae, and
4. enhance production management, quality control and evaluate performance.

## 2.2 Contents

The project included establishment of suited broodstocks, experiments to induce maturation in farmed and wild female and male eel, produce viable eggs and yolk-sac larvae, and establish culture conditions for larvae. The following areas were emphasised:

- **Broodstock:** Establishment of criteria for selection of mother fish and development of improved diets for the female broodstock in order to obtain a better egg and larvae quality.

Ultrasound scanning was used as a quick and efficient method to monitor female eels in the experiments by measuring ovary size and development.

- **Hormones:** Development and test of products for induced maturation, including hormone producing cells (Zanter project) and hormone preparations, which override the natural inhibition of the eel's reproduction in our waters.
- **Maturation method:** Tests of products, methods and hormone dosage to enhance the development of egg and semen.
- **Stripping methods:** Tests of various treatments for final maturation of the eggs and production of semen in order to improve the quality of gametes and fertilisation success.
- **Fertilisation and embryo development:** Improvement of fertilisation procedures and culture conditions under incubation with special focus on increasing embryo survival.
- **Larvae culture:** Develop suitable containers for cultures and suitable fish feed for larvae and describe growth and quality of larvae.

## 2.3 Project participants

In the project, knowledge institutes and the aquaculture industry collaborated on the integration of research areas, development of methods and technology and practical know-how using an applied approach. The various partners contributed to different tasks based on their field of expertise:

**Technical University of Denmark, National Institute for Aquatic Resources (DTU Aqua)** has extensive experience in project management and coordinated the earlier projects ROE II and III, which related to reproduction of eels in culture. Relevant research qualifications range from aquaculture and recirculation systems, technology development, experimental design etc., to nutritional requirements of fish, broodstock diets, fish reproduction, embryonic and larval development, and live and inert larval feed. The **National Food Institute (DTU Food)** contributes expertise in relation to fish nutrition and broodstock feed formulation.

**Danish Eel Farmers Association (DEFA)** has a broad knowledge about eel farming practice using recirculation systems including practical know-how and knowledge about growth, disease control, etc. DEFA's consultant participated in the previous DTU Aqua projects on eel reproduction involving experiments and data recording. The DTU eel research facility is located at Lyksvad Fish Farm and the owner of Lyksvad Fish Farm, Mogens Larsen, who rents out the buildings, also manages the alarm systems of the main facility. Oluf Sørensen, Stensgård Eel Farm, contributes by farming broodstock for the experiments using separate rearing systems and diets produced in the project.

**Billund Aquaculture Service (BA):** An international company that produces aquaculture systems for rearing of different fish species. BA has technical staff and expertise to build and enlarge the research facility. The consultant participated in previous DTU Aqua projects on eel reproduction with a central role in the experiments. BA's consultant guided the rebuilding and enlargement of the facility to accommodate mother fish, egg and larval cultures etc. The consultant contributes in the rearing of broodstock, reproduction experiments and larval production in the project.

**University of Copenhagen, Faculty of Life Sciences, (KU-Life):** Expertise within veterinary image diagnostics and image analysis. Ultrasound scanning is attractive as a non-invasive and non-lethal method to observe the development on an individual basis. The project participants from

KU-LIFE have contributed in previous projects with an efficient and careful method for monitoring individuals and thereby reducing the number of experimental animals. The KU-Life participants contribute with scanning and image analysis of female eels in the three experimental series.

**BioMar:** Danish fish feed company with international expertise in the production of fish feed. BioMar (before fusion DanaFeed) has contributed in previous projects with production of feed for mother fish and the development of larval feed. BioMar's consultants contribute to broodstock and larval feed development based on extensive knowledge about eel feed and formulation of fish diets in general.

**Bioneer:** Expertise in biotechnology and relevant experiences span from cell cultures and production of hormones to pharmaceutical knowledge of development of self-dosing hormones. Bioneer is essential for the development of new hormone preparations and is partner in the EUREKA project "Zanter". The Bioneer participants supply expertise within bioengineering (biomedical and biotechnological area) to explore the opportunities to develop novel hormone products.

## 2.4 Zanter-project

The project integrates funding of the Danish part of a bilateral cooperation between the SME ZF-Screens and Leiden University in the Netherlands, and DTU Aqua and Bioneer in Denmark. This project lead by ZF-Screens had the objective to develop and explore novel hormonal treatments potentially replacing salmon or carp pituitary extract presently used in the induction of female maturation. The Dutch partner intended to test hormone producing cell implants, while the Danish partners included the bioengineering company Bioneer with the intension to produce eel hormone using recombinant gene technology. The Zanter project proposal obtained the EUREKA label for innovation, but research was conducted in two bilaterally funded projects.

# 3 Implementation of the project

## 3.1 Tasks

The various partners provided staff and added expertise to implement the project and conduct the reproduction experiments. The work planned in the project was divided into the following tasks:

**Task 1: Implementation of full scale experiments.** The experimental studies in the project were carried out at the DTU Aqua research facility established at Lyksvad Fish Farm. The research facility including broodstock recirculation systems, and incubation and larval rearing facilities provided the basis for the experiments as well as office and laboratory facilities used to support the work. The research facility was managed by DTU Aqua in collaboration with DEFA and BA.

**Task 2: Development and testing of feed for broodstock eel.** Improved broodstock diets developed by BioMar and DTU Aqua were tested. The broodstocks fed different diets were cultured at either Lyksvad Fish Farm or Stensgård Eel Farm. The farmed broodstocks were applied in the experiments at the Lyksvad facility and were sampled during culture as well as during experiments.

**Task 3: Monitoring of breeding fish at the start and during treatment.** Female size and condition are key parameters used in the selection of broodstock. At the onset and during the course of the experiments ultrasound was used to determine the ovarian size and develop a non-invasive method to monitor the efficacy of treatments. The task was carried out by veterinarians from KU-Life, working with image analysis and CT scans of eels. The latter was conducted in order to investigate the reallocation of resources to ovarian developments. The ultrasound method was applied to identify good responders and allow for elimination of poor responders early in the treatment inducing female maturation.

**Task 4: Development and testing of alternative hormone replacement therapy and treatments**. The present treatment to induce maturation of eels is a hormone extract originating from salmon or carp pituitaries. The product originates from another species with a sub-optimal non-homogeneous hormone composition and an inherent danger of disease transfer. Therefore, the partner Bioneer explored alternatives to produce eel hormones using recombinant gene technology and a precursor of gene producing implants produced by Dutch collaborators was tested.

**Task 5: Optimization of methods of fertilisation, incubation of eggs and hatching of larvae.** The fertilisation tests included monitoring egg and sperm quantity and quality, standardizing sperm dosage in fertilisation procedures, methods for incubation of fertilized eggs and hatching of larvae. Rearing procedures and techniques to enhance survival of embryos were improved by DTU Aqua and BA through small to medium scale experiments and successfully implemented in the full scale experiments.

**Task 6: Development of larval tanks and the establishment of larval cultures.** Eel larvae are only little developed when they hatch two days after fertilisation. The development of e.g. eyes, jaws and the digestive system take place through the yolk-sac stage that lasts around 12 days at 20°C before they reach the stage where they can start feeding. During this period and also later, the larvae are sensitive to turbulence, air bubbles and light. As part of this task a new type of larval rearing container was developed in collaboration between DTU Aqua and BA. In addition, BioMar and DTU Aqua developed a first inert diet for larvae to be tested in future experiments.

**Task 7: Operational and technical development of experimental facilities.** The research facility has a permanent staff from DTU Aqua, BA and DEFA, who performed the full scale experiments and tasks in the project in close collaboration with the project leader and DTU Aqua staff taking part in the work during temporary intensive periods. The technical and practical enhancement of the facility included temperature control systems to regulate temperature in broodstock systems, change of light sources to imitate natural day light and a PLC system that registers physical and chemical conditions. Laboratory conditions were also improved.

## 3.2 Time schedule and milestones

The project period started 1st October 2008. Different improvements including establishment of a laboratory and enhancement of rearing facilities were made during the period as planned. Broodstock culture on different feeds was established and three experimental series were conducted: The 1$^{st}$ experimental period spanned from June 2009 to December 2009. The 2$^{nd}$ and 3$^{rd}$ series were conducted from January 2010 to June 2010 including a smaller facility for wild eels

located at BA. In addition, a shorter experiment related to the Zanter project was carried out to test precursors for hormone implants. According to the original plan the project should have been completed 31st March 2010 but was granted an extension until the end of the year 31st December 2010.

**Milestones**

**M 1.1:** Experimental series accomplished and reported to project participants.
**M 2.1:** Development of improved mother fish feed for eels accomplished.
**M 2.2:** ROE lll and new mother fish feed tested and reported.
**M 3.1:** Scanning method to select mother fish on site and a measure for response was incorporated, considering the possibility of excluding females that did not respond to treatment.
**M 4.1:** Production technology to produce FSH and LF has been developed.
**M 4.2:** Evaluation of ZF-implants, alternative maturation hormones (FSH and LF), SPE treatment and different final maturation hormones.
**M 5.1:** Development of methods to calculate fertilisation success and survival rates as well as an evaluation of egg and embryo quality.
**M 5.2:** Standard protocols have been developed for storage of semen medium, fertilisation and incubation.
**M 6.1:** Larvae feed developed and tested.
**M 6.2:** Larvae tanks developed and tested.
**M 6.3:** Protocols for culturing larvae and methods to determine survival and growth were developed.
**M 7.1:** PLC system installed, fume hood established and security conditions authorized.

# 4 Project results

## 4.1 Task 1: Implementation of full scale experiments

*Jonna Tomkiewicz, Lars Tybjerg, Peter Lauesen, Christian Graver, and Sune Riis Sørensen*

### 4.1.1 Introduction

A total of three full scale experimental series i.e. REEL A, B and C were conducted during the project period at the DTU experimental facility located on Lyksvad Fishfarm. The experimental protocols were developed by the project group integrating schemes for development and test of broodstock feed, as well as methods and procedures for hormonal treatment, fertilisation, incubation and larval rearing. Similarly plans for sampling and monitoring, and a database to record data were established. Various parameters were tested in the three experiments including parameters such as origin and size of broodstock eels, broodstock feeds, hormonal treatments and fertilisation methods. Methods and technology developed during previous projects: Artificial Reproduction of Eels (ROE lll) and the extension: Artificial Reproduction of Eels - Larval Culture (ROE lll-LC), were used as the starting point in order to consolidate and further improve methods and technology. Standardised methods to evaluate processes and monitor production were

established in order to enhance analysis and interpretation of results. In addition, a smaller experiment was conducted to test the viability of cell implants as part of development of hormone producing implants, which can be used as an alternative treatment to induce maturation in broodstock eels. The performed test of viability of cells developed by collaborators in the Zanter project is presented and discussed in relation to Task 4 (Section 4.4). Protocols and sampling plans elaborated by project participants and the established protocols were implemented at the experimental research facility by DTU Aqua, BA and DEFA. Samples and data were provided to participants. Results of the experiments were analysed and results evaluated (Tomkiewicz et. al. *(In prep.)*).

**Milestones**

**M 1.1:** Experimental series - designed, accomplished and reported to project participants. (Section 4.1.2).

## 4.1.2 Materials and methods used in full scale experiment

**Material, methods and trials**

The three full scale experimental series included two farmed female broodstocks and one wild caught broodstock of female silver eels. In each series, farmed or wild broodstock females were held in separate recirculation systems each including two containers (each 300 l). Male broodstocks were held in a similar four container system. The farmed broodstocks originated from different eel farms i.e. Lyksvad Fishfarm and Stensgård Eelfarm, where they were fed standard or enhanced diets (Section 4.2). The reproduction experiments were conducted at DTU Aqua's experimental facility located on Lyksvad Fishfarm.

For all series, standard physical-chemical parameters at the experimental research facility were monitored and controlled during the maturation and fertilisation period. Table 4.1.1 shows the aimed values and range of physical-chemical parameters in experiments.

**Table 4.1.1.** Aimed values and range of physical-chemical parameters in experiments.

| Parameters | Conditions |
|---|---|
| Saltwater | Artificial saltwater (35 ‰) and natural seawater |
| Water temperature | 19.5 – 20.5 $^{o}$ C |
| Oxygen | 90 – 100 % |
| pH | 8.0 – 8. 2 |
| Water chemistry | $NH_3/NH_4^+$< 0.01 mg/l<br>$NO_2$ <0.1 mg/l<br>$NO_3$ < 50 mg/l |
| Light regimes: | |
| REEL series A and C | No light except during working hours |
| REEL series B | Imitating natural daylight, dusk/dawn; local diurnal periodicity |

*REEL Series A*

This series was based on female eels farmed at Lyksvad Fish Farm and offered two different diets. One group of female eels received standard eel feed and a second group of females received an improved feed developed for female eel broodstock in a previous project (Støttrup et al. 2012). The feed used in REEL was produced by BioMar, and the first feeding experiment started in 2008. Female broodstocks of the same origin, but fed the two different diets, entered reproduction trials after a feeding period of 20 weeks. The experimental period from induction of maturation to the end of the larval rearing trials, spanned from June 2009 to December 2009.

A female size in the weight interval from 600-800 grams was aimed at for the reproduction experiments. Eels were transferred from the fish farm to the research facility and acclimatized according to a standard protocol (1-2 weeks). Eels were divided into groups of 2x10 eels per recirculation system and reared under standard physical-chemical conditions (Table 4.1.1) and different treatment schemes (Table 4.1.2) during the maturation period. A total of 120 females were included in this series. At the start of maturation, morphometric measures were taken and each fish tagged. The females in the 12 groups were weighed weekly and injected with salmon pituitary extract (SPE) weekly. Different SPE dosages and addition of vitamins were tested. The hormone Deoxycortesterone (DOC) was used to induce final maturation. The experimental tests conducted are summarized in Table 4.1.2.

Two groups of 20 male eels were selected from the same location and acclimatized using standard protocol resembling the female protocol. Morphometric measures were obtained as described for the females. The two groups were started at various time points to ensure sufficient semen production during the fertilisation period. Males were weekly injected with 200 IU human chorionic gonadotropin (hCG). Sampling plans including 0-sampling, ultrasound scanning, etc. for the female broodstock was developed and implemented. In REEL A, a new fertilisation protocol was developed and tested.

**Table 4.1.2.** Tests of effects of female feed, hormonal treatments etc. conducted in REEL experimental series A. Circles indicate treatment parameters for each group.

| **REEL Series A** | **Groups** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Parameters** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** |
| Diet: | | | | | | | | | | | | |
| Standard feed | | O | | O | | O | | O | | O | O | O |
| Improved broodstock feed | O | | O | | O | | O | | O | | | |
| Vitamin C and D | | | O | O | | | | | | | | |
| Hormonal induction: | | | | | | | | | | | | |
| 25 mg/kg/week SPE | O | O | | | | | | | | | | |
| 18.25 mg/kg/week SPE | | | O | O | O | O | | | | | O | O |
| 12.5 mg/kg/week SPE | | | | | | | O | O | | | | |
| 12.5 -25 mg/kg/week SPE | | | | | | | | | O | O | | |
| Final maturation: DOC | O | O | O | O | O | O | O | O | O | O | O | O |

Application of a seminal dilution medium developed by Japanese researchers and modified by Asturiano et al. (2003, 2004) was compared with the standard protocol using fresh semen in *in vitro* fertilisation. The aim was to standardise sperm addition and increase fertilisation rates through a more uniform mixing of semen and eggs. The development of a method to quantify fertilisation rates was initiated as well as monitoring embryonic and larval quality using photo documentation. Task 5 reports the results (Section 4.5)

*REEL Series B*

Female eels in this series originated from two independent locations. In total, 110 females were selected from Stensgård Eel Farm and 20 female eels from Lyksvad Fish Farm to evaluate differences among females from different sources and differing, in particular, in size. Both groups were reared on standard eel feed (BioMar). The experimental period spanned from January 2010 to June 2010.

The female eels selected for the experiments were in the weight range from 450-650 grams. Procedures for transfer to the research facility, acclimatisation, morphometric measures and tagging were similar to REEL Series A. 130 eels were equally distributed among 13 groups. The experimental set-up of REEL Series B included a test of different hormone batches of SPE and product types in addition to treatments and source and size of eels. Furthermore, two different hormones DOC and 17,20β-dihydroxy- 4-pregnen-3-one (DHP) used to induce final maturation were tested. The experimental setup is summarized in Table 4.1.3.

**Table 4.1.3.** Tests of effects of female broodstock origin and treatments performed in REEL experimental series B. In addition, the activity of SPE Lot 1 and 2 referring to different shipments of SPE was tested. Key treatment parameters are indicated for each group.

| **REEL Series B** | **Groups** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Parameters** | **A** | **B** | **BX** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** |
| Broodstock origin: | | | | | | | | | | | | | |
| Stensgård Eel Farm | O | O | O | O | O | | | O | O | O | O | O | O |
| Lyksvad Fish Farm | | | | | | O | O | | | | | | |
| Diet: Standard feed | O | O | O | O | O | O | O | O | O | O | O | O | O |
| Hormonal induction: | | | | | | | | | | | | | |
| 12.5 -25 mg/kg/week SPE (Lot 1) | | | | O | O | O | O | O | O | | | | |
| 18.25 mg/kg/week SPE (Lot 2) | O | | | | | | | | | | | | |
| 18.25 mg/kg/week SPE (Lot 1) | | O | | | | | | | | O | O | O | O |
| 18.25 mg/kg/week SPE (Lot 2) | | | O | | | | | | | | | | |
| Frequency: | | | | | | | | | | | | | |
| Weekly injection | O | O | O | O | O | O | O | O | O | O | O | O | O |
| Final maturation: | | | | | | | | | | | | | |
| DHP | | | | | | O | | O | O | | | O | O |
| DOC | O | O | O | O | O | | O | | | O | O | | |

Similar to REEL Series A, males were transferred to the experimental facility applying standard conditioning procedures before start of hormonal injection. 200 IU of hCG was injected per male on

a weekly basis and two groups were started at successive dates with a time lapse ensuring spawning readiness and the sperm production needed to supply high quality sperm during the entire fertilisation period.

Ultrasound scanning was conducted to monitor ovarian development, prior to onset of experiments as well as during the experiments: Sacrifice and sampling of female tissues at the onset (0-sampling) as well as sampling of eggs from stripped females were conducted for analysis of lipid content and composition. The application of a seminal dilution medium enhanced fertilisation success in the fertilisation procedures tested in series A. Consequently, this procedure was implemented as standard in series REEL B and additional trials comparing application of fresh sperm and diluted sperm in fertilisation procedures were conducted. The monitoring procedure developed during REEL A to assess fertilisation success was implemented as a new routine in the experiments.

*REEL Series C*

These experiments used wild female eels from a lake in northern Jutland in Thy National Park called Lake Vandet. The eels were obtained in November 2009, in total 22 migrating silver eels. These eels followed the same acclimatisation, measuring and tagging procedure as described for REEL Series A and REEL Series B. The female eels were divided into two groups and subjected to two different hormonal treatments. DOC was applied for final maturation. The experimental setup is summarized in Table 4.1.4. Eggs from stripped females were sampled for lipid analysis.

Farmed male eels from Stensgård Eel Farm were applied as broodstock in the experiment. Protocols for male maturation, fertilisation and egg monitoring were similar to REEL Series B. The experimental period from start of induction of maturation until the end of incubation and larval rearing spanned from January 2010 to July 2010.

**Table 4.1.4.** Experimental tests performed in series REEL 1C indicating key treatment parameters for each Group.

| **REEL 1C** | **Groups** | |
|---|---|---|
| **Parameters** | **M** | **N** |
| Hormonal induction:<br>12.5 --> 25 mg/kg/week SPE | O | |
| 18.25 mg/kg/week SPE | | O |
| Frequency:<br>Weekly injection | O | O |
| Final maturation: DOC | O | O |

## 4.1.3. Results and discussion of experiments

Table 4.1.5 provides the overall results of the experimental series REEL A, B and C. The experimental series REEL A and B used farmed fishes and REEL C wild female eel. In series REEL A and C around 50 % of the females initiated in the experiments were successfully stripped, which corresponded to results obtained in previous experiments (e.g. Tomkiewicz, 2008;

Tomkiewicz & Jarlbæk 2008; Tomkiewicz & Sørensen, 2008), while the proportion of females successfully stripped in REEL B was considerably lower (15 %). The latter was caused by a high proportion of non-responding females (Table 4.1.5). A high proportion of non-responding females was also the main cause of failure in females in series REEL A, where the female broodstock as in series B were farmed eels. In contrast, the main loss of potential breeders in series C was caused by death or disease of the female eels (27%), which is considerably higher than observed in the farmed eels.

**Table 4.1.5.** Results of the experimental series REEL A, B and C as a total and in percent independent of treatments within Groups.

| | REEL A | | REEL B | | REEL C | |
|---|---|---|---|---|---|---|
| **Female eels** | ***No. Started*** | ***% of started*** | ***No. started*** | ***% of started*** | ***No. started*** | ***% of started*** |
| Broodstock | 120 | | 130 | | 22 | |
| Dead/sacrificed | 13 | 11% | 11 | 8% | 6 | 27% |
| Non-repsonding | 38 | 32% | 93 | 72% | 2 | 9% |
| Spontaneous ovulation | 5 | 4% | 0 | 0% | 2 | 9% |
| No ovulation | 2 | 2% | 0 | 0% | 1 | 5% |
| Overripe | 0 | 0% | 7 | 5% | 0 | 0% |
| Stripped | 62 | 52% | 19 | 15% | 11 | 50% |

| | REEL A | | REEL B | | REEL C | |
|---|---|---|---|---|---|---|
| **Female eels** | ***No. started*** | ***% of started*** | ***No. started*** | ***% of started*** | ***No. started*** | ***% of started*** |
| Stripped | 62 | | 19 | | 11 | |
| Fetrtilised | 29 | 47% | 5 | 26% | 9 | 82% |
| Embryos | 8 | 13% | 2 | 11% | 6 | 55% |
| Larvae | 4 | 6% | 1 | 5% | 6 | 55% |

All series independent of broodstock source and treatment produced fertilised eggs, viable embryos and larvae (Table 4.1.5 and Fig 4.1.1).

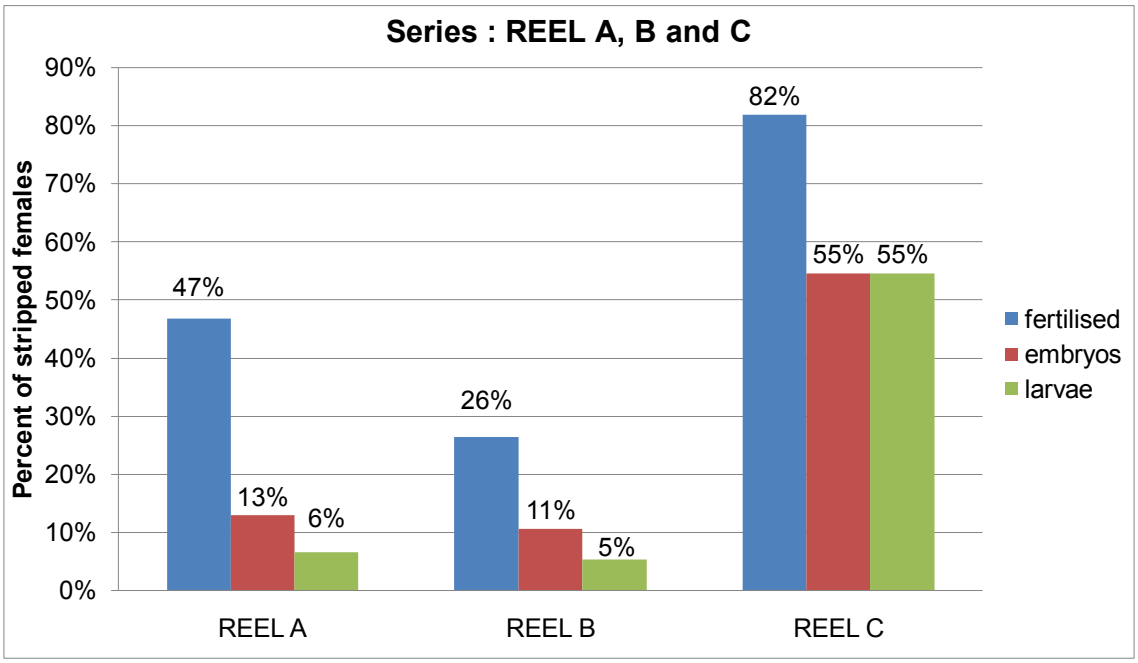


**Figure 4.1.1.** Female success rate in the three series of experiments expressed as per cent females successfully stripped and the related success rate obtaining embryos and larvae.

The number of fertilised eggs per batch differed significantly and this also applied to the number of larvae hatched per batch. Table 4.1.6 shows the number of larvae hatched per female in the three experimental series. In Series A, the four females producing larvae had received different diets; three out of the four had received the improved diet (groups A, C and I), while one female received standard diet (group D). The numbers hatched were low and the survival of the larvae limited to a few days. In Series B, only one female produced larvae. In contrast, the numbers hatched and survival of the larvae was extraordinarily high in series C with mass hatching in three out of six successful batches. The larvae from the three mass hatchings completed the yolk-sac stage around day 12 and entered first feeding experiments. The experiments thereby consolidated the results from ROE III, where larvae from mass hatching lived up to 18 days. The longest living larvae in ROE III originated from farmed eels.

In these experiments, the wild eels proved to be successful in the experimental reproduction. The long term strategy is to use farmed eels for a self-sustained reproduction in aquaculture, however wild eels are useful as a model for comparison. This includes the lipid composition of different body parts before treatment, as all wild eels are silver eels and thus should have the right composition of a potential female spawner. Also the allocation of fatty acids and other changes among body parts are important as is the lipid composition of the eggs. The eggs of the wild eels play a major role in the evaluation of the quality of the broodstock feed, the hormonal treatment as well as the egg quality and viability. The results of experiments on broodstock diet, different hormonal treatments and fertilisation, embryonic and larval rearing success within the series are further elaborated in the following sections.

**Table 4.1.6.** Number of larvae hatched per female and their longevity (post hatch) in the experimental series REEL A, B and C in relation to treatments.

| Series | Group | Female ID | Date of stripping | Larvae *No.* | Life span *Days* |
|---|---|---|---|---|---|
| R1A | A | A7DE6 | 12-sep-09 | <100 | 2 |
| R1A | I | A7A03 | 20-okt-09 | 10 | 1 |
| R1A | C | A655B | 12-sep-09 | < 100 | 3 |
| R1A | D | A63F3 | 24-sep-09 | < 10 | 2 |
| R1B | E | A6416 | 13-maj-10 | 6 | 2 |
| R1C | M | A83F3 | 16-maj-10 | no data | 5 |
| R1C | M | A746D | 16-maj-10 | 200,000 | 20 |
| R1C | M | A7432 | 02-maj-10 | 100,000 | 15 |
| R1C | M | A6EC5 | 13-apr-10 | <100 | 14.5 |
| R1C | N | A648B | 21-maj-10 | < 100 | 3 |
| R1C | N | A635D | 05-maj-10 | 100,000 | 5.5 |

## 4.2 Task 2: Development and test of female broodstock diets

*Josianne G. Støttrup, Jonna Tomkiewicz, Lars K. Holst, Charlotte Jacobsen, Lars Tybjerg, Peter Lauesen, and Christian Graver*

### 4.2.1 Introduction

Farmed broodstocks were started and cultured at Lyksvad Fish Farm on two different diets. The diet experiments included two different feed types: standard feed and the improved broodstock diet, JD1, developed during previous projects was tested (Støttrup et al. 2012). Later during the project, the special feed for female broodstock was further developed and tested in collaboration with the feed company BioMar. This feed, JD2, is a further development of the existing feed, JD1. The diets produced and a standard feed were fed to three groups of broodstock farmed at the commercial farm, where the research facility is located, i.e. Lyksvad Fish Farm. The standard feed is the BioMar eel diet applied in commercial eel farming (Danex 2848 2 mm).

**Milestones**

**M 2.1:** Development of improved mother fish feed for eels - accomplished (Section 4.2.2).

**M 2.2:** ROE lll and new broodstock feed – developed, tested and reported (Section 4.2.3).

### 4.2.2 Development of broodstock feed

Levels of protein, fat and ash in the new and traditional feeds were similar. The JD1 feed had additional essential PUFAs and asthaxanthin. The new feed, JD2, had a different composition.

Krill meal, phospholipids and mono-calcium phosphate were introduced in this diet and the levels of some vitamins were increased. Higher vitamin C (app. 55%) and more than double the amount of vitamin E content was used in the JD2-diet compared to in the standard diet. The PUFA content, in particular ARA, EPA and DHA differed in all three diets. All three diets were formulated and produced by BioMar.

### 4.2.3 Broodstock culture and tests

#### Establishment of broodstocks

*REEL Series A*

In 2008, the first farmed broodstocks were started and cultured at Lyksvad Fish Farm. Two containers applying different feed types were applied and eels were fed several times a day, following the standard feeding cycle at the farm. The feed tested was formulated during ROE II included two different feed types:

1. Standard feed: BioMar (Danafeed) DAN-EX 2848
2. Improved broodstock feed 1- JD1: DHA/EPA/ARA/ASTA

During the feeding period samples of feed and broodstock were obtained at different intervals. At start of Series A, broodstock females in the weight interval of 600-800g were selected and transferred to the experimental facility. Experiments were conducted using this broodstock.

Sampling of feed and female eels was conducted during the feeding experiment and following the experiments the samples were analysed for lipid composition. In Series A, it was detected that some feed samples had deteriorated. This may have been due to too large quantities produced and storage in a non-cooled place leading to rancidification of the feed. Furthermore, problems with the water quality had been observed in the systems where the broodstocks were cultured. Given the possible sources of error, it was decided not to rely on this experimental series for the test of feed types and as a consequence no analyses of the samples of female eels obtained during the experiments were made. Instead these two feed types were both included in the next tests of feeds leading to the broodstocks for the experimental series B. The feed was produced in smaller quantities and stored cold until use. The succeeding feeding experiments and broodstock culture were relocated to Stensgård Eel Farm to avoid problems with the water quality.

*REEL Series B*

During October 2009 a new broodstock was set up at Stensgård Eel Farm. A total of 720 kg eels were selected with an average weight of 475 g. They were selected based on healthy appearance i.e. no visible signs of wounds and other abnormalities. The new broodstock originated from the 2006-2008 glass eel catches. The eels were randomly distributed in three separate - but physically connected – water basins, designed for three different feeding trials. Rearing conditions did not differ from the general conditions at the farm and no specific records were obtained.

Feeding was initiated with feed produced at BioMar and included three different feed types:

1. Standard feed: BioMar (Danafeed) DAN-EX 2848
2. Improved broodstock feed 1 - JD1: DHA/EPA/ARA/ASTA
3. Improved broodstock feed 2 - JD2: DHA/EPA/ARA/ASTA + vitamin

Eels were – as described for the first broodstock - fed several times a day, following the standard feeding cycle at the farm. As a consequence of low appetite among the broodstock females, a portion of small eels was added in order to stimulate their appetite. The eels were fed until June 2010 and entered the process of maturation at the DTU experimental facility and were used in the first series of the EU-project PRO-EEL (Støttrup et al., *In prep*).

### Sampling

During the feeding period fish were sampled according to the plan (Table 4.2.1) to document and evaluate the effects of the feeding. At the same sampling time, feed samples were taken and sent to DTU Aqua and BioMar for analysis.

Changes in lipid in female broodstock were monitored during 5 samplings. The 0 sample is the original fish before being separated and fed different diets (FF). The FFS series were fed the standard or traditional eel diet (Danex 2848 2 mm). The remaining fish were fed either JD1 (FF1 fish) or JD2 (FF2 fish) and sampled at 8, 16, 24 and 38 weeks of feeding. The broodstock provides the basis of the first experiments in the PRO-EEL project and sampling in week 38 provided the 0-samples for these experiments.

**Table 4.2.1.** Broodstock sampling scheme. The sampling weeks is seen in the left column followed by the number and type of samples for both DTU Aqua and BioMar A/S.

| Sampling time (weeks) | Fish No. | Samples | |
|---|---|---|---|
| | | DTU Aqua | BioMar A/S |
| 0 | 5 | Liver for analysis | Ovary for analysis |
| 8 | For each feed type: 5 | Muscle for analysis<br>Ovary for histology | |
| 16 | For each feed type: 5 | Ovary for analysis | |
| 24 | For each feed type: 5 | | |
| 38 | For each feed type: 5 | | |

### Results

As expected the broodstock lipids reflected the dietary lipids with time. ARA content became highest and EPA became lowest in those females feeding on JD1, which is the diet with the highest ARA content and lowest EPA content. As shown in Figure 4.2.1 and 4.2.2, both test diets significantly changed the ratios of EPA:ARA, due to the increase in ARA in both diets. The low EPA content in JD1 also resulted in an increasing change in DHA:EPA ratios with time, which was more pronounced than in broodstock feeding on the JD2L diet. The two diets (JD1 and JJNL) were enriched with asthaxanthin. Analysis of gonads after 16 weeks of feeding with all 3 diets showed no detectable levels of asthaxanthin.

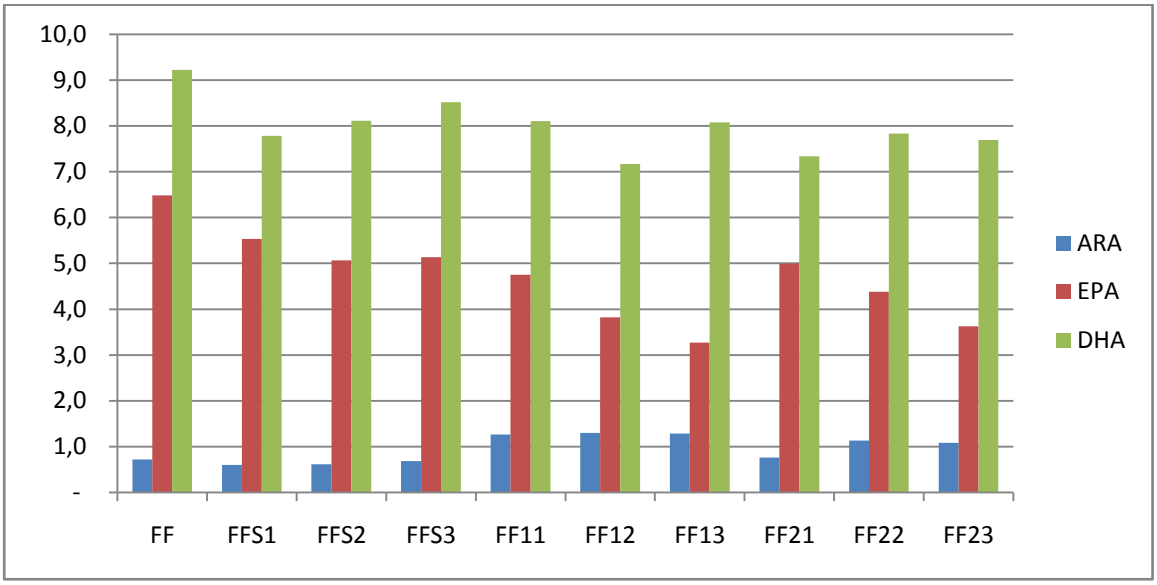


**Figure 4.2.1.** Fatty acid analysis of broodstock Series B. Levels (% of NL lipids) of ARA, EPA and DHA in eels fed the traditional feed, Danex 2848 (FF and FFS series), JD1 diet (FF1 series) and JJNL diet (FF2 series) for 0 (FF), 8 (1), 16 (2) and 24 weeks (3). Thus FF12 denotes JD1 diet for 16 weeks.

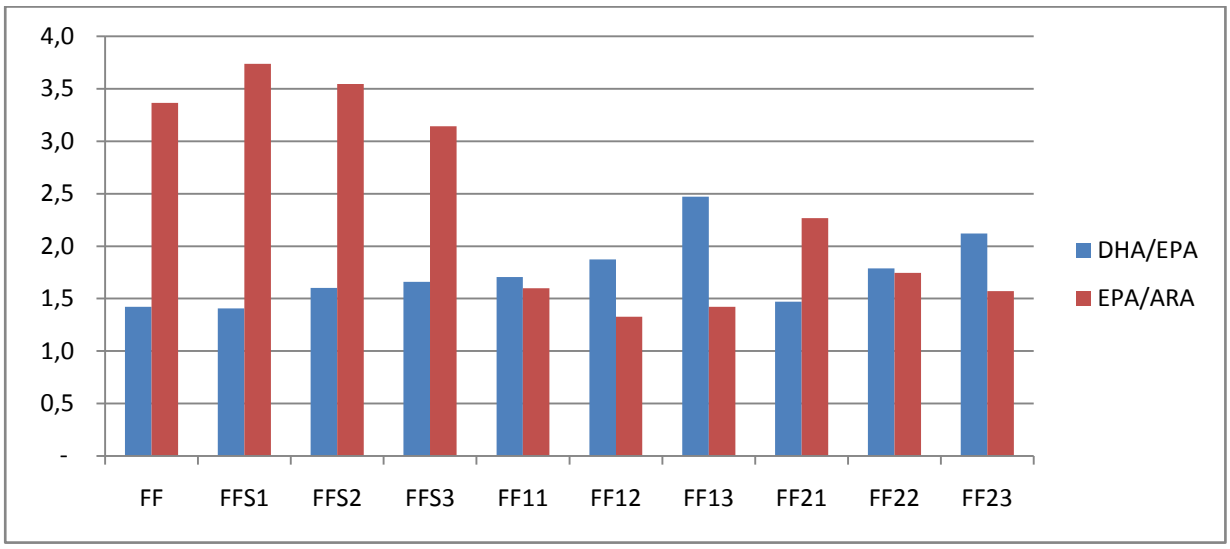


**Figure 4.2.2.** Fatty acid analysis of broodstock Series B. Ratios of DHA/EPA and of EPA/ARA in eels fed the traditional feed, Danex 2848 (FF and FFS series), JD1 diet (FF1 series) and JD2 diet (FF2 series) for 0 (FF), 8 (1), 16 (2) and 24 weeks (3). Thus FF12 denotes JD1 diet for 16 weeks.

**Table 4.2.2.** List of eggs sampled for lipid analysis with female history. Origin refers to the broodstocks from Stensgård Eel Farm, Lyksvad Fish Farm and Lake Vandet. The options for Larvae TRUE or FALSE indicate whether larvae hatched = TRUE and no larvae hatched = FALSE.

| Project | Start date | ID | Batch | Saltwater acc. weeks | Origin | Weight g | Length cm | Larvae obtained |
|---|---|---|---|---|---|---|---|---|
| REELB | 19-Jan-10 | A7895 | A | 4 | Stensgård | 592 | 66 | FALSE |
| REELB | 06-Jan-10 | A6B12 | C | 2 | Stensgård | 594 | 66 | FALSE |
| REELB | 06-Jan-10 | A79A6 | C | 2 | Stensgård | 467 | 59 | FALSE |
| REELB | 06-Jan-10 | A6416 | E | 2 | Lyksvad | 882 | 74 | TRUE |
| REELB | 06-Jan-10 | A83D5 | E | 2 | Lyksvad | 833 | 74 | FALSE |
| REELB | 06-Jan-10 | A850F | E | 2 | Lyksvad | 744 | 69 | FALSE |
| REELB | 06-Jan-10 | A676F | F | 2 | Lyksvad | 871 | 70 | FALSE |
| REELB | 06-Jan-10 | A6F1D | F | 2 | Lyksvad | 774 | 70 | FALSE |
| REELB | 06-Jan-10 | A7469 | F | 2 | Lyksvad | 771 | 71 | FALSE |
| REELB | 06-Jan-10 | A8A01 | F | 2 | Lyksvad | 710 | 67 | FALSE |
| REELB | 06-Jan-10 | A8A29 | F | 2 | Lyksvad | 1365 | 79 | FALSE |
| REELB | 06-Jan-10 | A8B37 | F | 2 | Lyksvad | 661 | 67 | FALSE |
| REELB | 06-Jan-10 | A905A | F | 2 | Lyksvad | 632 | 67 | FALSE |
| REELB | 06-Jan-10 | A8A40 | G | 2 | Stensgård | 515 | 61 | FALSE |
| REELB | 06-Jan-10 | A6A17 | I | 2 | Stensgård | 497 | 64 | FALSE |
| REELB | 06-Jan-10 | A694C | K | 2 | Stensgård | 547 | 65 | FALSE |
| REELB | 06-Jan-10 | A63E9 | L | 2 | Stensgård | 508 | 63 | FALSE |
| REELB | 06-Jan-10 | A7933 | L | 2 | Stensgård | 616 | 66 | FALSE |
| REELB | 06-Jan-10 | A7E13 | L | 2 | Stensgård | 462 | 62 | FALSE |
| REELC | 07-Jan-10 | A6700 | M | 4 | L. Vandet | 558 | 66 | FALSE |
| REELC | 07-Jan-10 | A746D | M | 4 | L. Vandet | 750 | 71 | TRUE |
| REELC | 07-Jan-10 | A635D | N | 4 | L. Vandet | 540 | 61 | TRUE |
| REELC | 07-Jan-10 | A6462 | N | 4 | L. Vandet | 390 | 62 | FALSE |
| REELC | 07-Jan-10 | A6EC5 | M | 4 | L. Vandet | 570 | 68 | TRUE |
| REELC | 07-Jan-10 | A7432 | M | 4 | L. Vandet | 636 | 70 | TRUE |

*Lipids in eggs from broodstock of different origin – series B and C*

Egg samples for fatty acid analysis were taken from broodstock of different origins (Table 4.2.2). The females were either farmed fish (9 from Stensgård Eel Farm and 10 from Lyksvad Fish Farm) fed standard eel diet, or wild fish from Lake Vandet (6 fish). The farmed fish measured 67.4+4.9 cm and weighed 686.4+212.7 g, while the wild fish measured 66.3+4.1 cm and 574.0+118.4 g.

Only one of the farmed fish provided viable eggs whereas 4 out of 6 wild fish provided viable eggs that hatched into larvae. The fatty acid results showed significant differences in fatty acid distribution and levels of essential fatty acids between eggs from farmed and wild fish. These results will be used to further develop broodstock feed for farmed eel with the aim to alter content of eggs produced in farmed eel to mimic that in eggs produced from wild females.

## 4.3 Task 3: Monitoring of mother fish at start of experiment and during treatment

*Fintan McEvoy, Lene Buelund, Lars Tybjerg, Jonna Tomkiewicz, and Eiliv Svalastoga*

### 4.3.1 Introduction

Ovary size is of great importance when selecting parent fish and in this case ultrasound scanning was used to identify ovary size both at the beginning and during the experimental period. The task was carried out by the participants from KU-Life in collaboration with DTU Aqua, BA and DEFA. The method developed in the previous projects ROE II and ROE III was used to monitor response and development rates in order to compare treatments. The method was further elaborated during the project period.

#### Milestones

**M 3.1:** Scanning method to select mother fish on site and as a measure of treatment efficacy was developed and applied, including the opportunity of excluding females that do not respond to treatment.

### 4.3.2 Ultrasound scanning as a non-invasive monitoring method

#### Materials and methods

In previous projects the application of ultrasound scanning was developed and used as a method to monitor ovarian responsiveness to hormonal treatment. For responding eels the ovary will grow during maturation and this was documented by scanning the eels at regular intervals. Also non-responding eels could at an early stage be identified and subsequently excluded from the experiment, thereby reducing the workload for the staff at the facility and furthermore save costly hormone product.

In REEL 1B this method was applied for all female eels entering maturation. A total of four scanning sessions were made during maturation: at week 0, 6, 10 and 14 after start of the hormonal treatment.

Eels were caught with nets and slightly anaesthetized with Benzokain until motion ceased. One eel at the time was quickly transferred to the scanning equipment and scanning was performed 3 cm anterior of the genital pore, at the site of the liver and close to the eye. Eels were subsequently transferred to the water basins for recovery.

**Results**

Methods developed in previous eel experiments (ROE III and earlier projects) were used on site at the research facility to scan at the onset and during hormone treatment. The technique involved obtaining transverse scans at specific anatomical sites. Images were recorded on disc and later analysed. The protocol is considered to be largely stress free for the fish and when implemented correctly, takes two minutes per fish to perform.

Scans of the ovary from each fish were obtained at the start and every 2 weeks during the hormone treatment phase. In addition, scans of the liver (at the position of its maximal cross sectional area) and of the eye were also obtained.

Images were later reviewed and measurements of ovary and liver cross-sectional area and of eye diameter were made using calibration / measurement software in the ultrasound scanner.

Fish were grouped as having a successful outcome or not. Females that reached the stripping stage or a later stage were classed as successful. The Welsh two sample t-test was used to determine if there was a difference in ovarian cross sectional area between the two groups. In addition a linear model was used to determine the usefulness of ovarian cross sectional area, liver cross-sectional area and eye diameter at various time points, as predictors of success.

Significant statistical differences in ovarian cross sectional diameter were seen from the second scan session onwards ($P < 0.05$) or less. When ovarian and liver cross sectional area were used together with eye diameter on the two week scan data, in a linear model, ovarian cross sectional area contributed the most of the three as a predictor of a successful outcome, but no parameter alone made a statistically significant contribution. When the same model was used on data from the $6^{th}$ scanning session, ovarian diameter made a significant contribution ($P=0.006$) while eye diameter and liver cross section area did not contribute significantly to the model, provided ovarian cross sectional are included.

The data suggest that ovarian cross sectional diameter can be used as a predictor of a successful outcome at the time of the $6^{th}$ scan (after 12 weeks hormonal treatment). These results are encouraging and the use of ultrasound should be investigated further in repeat trials with greater group numbers and possibly, alternative success criteria.

## 4.4 Task 4: Development and trials of alternative hormone preparations and treatments

*Peter Ravn, Bjørn Holst, Lars Tybjerg, and Jonna Tomkiewicz*

### 4.4.1 Introduction

The most common method used to induce maturation in female eels is weekly injections of a hormone product made of freeze-dried pituitaries from migrating Pacific salmon (SPE) or carp (CPE). The product is however not homogenous but varies between lots in the amount of active hormone. Neither is the hormone composition in these pituitaries optimal for the repeated treatment during induced maturation as it does not follow the natural hormonal cycle of fish during development. Therefore, the potential for a production of eel hormones by means of recombinant

gene technology was investigated. This task was carried out by Bioneer in collaboration with DTU Aqua and is described in 4.4.2 Production technology to produce FSH and LF.

Another method was presented by the ZF-Screens and Leiden University as the Dutch contribution to the Zanter project. The weekly injection of pituitary extracts implies that every eel must be handled manually each week, thereby increasing the risk of stress. As an alternative to the weekly hormonal stimulation, ZF-Screens and Leiden University work on developing cell implants that can be injected in the abdominal cavity and produce hormones over a prolonged period of time. These cells, ZF-implants (patented by ZF-Screens) uses zebrafish cells that produce eel hormones using recombinant technology. The test is described in 4.4.2 Evaluation of ZF-implants, alternative maturing hormones (FSH and LF), SPE treatment and different final maturation hormones.

**Milestones**

**M 4.1:** Production technology to produce FSH and LF has been developed (Section 4.4.2).

**M 4.2:** Evaluation of ZF-implants, alternative maturing hormones (FSH and LF), SPE treatment and different final maturation hormones – precursor tested (Section 4.2.3).

## 4.4.2 Production technology to produce FSH and LF

**Introduction**

The hormones, which are considered important for artificial maturation of eels, are primarily follicle stimulating hormone (FSH) and Luteinizing hormone (LH). Access to pure FSH and LH would enable development of more consistent hormone preparations, which could be optimized to mimic the natural hormone changes in maturing eels. Furthermore, use of pure hormone preparations would add insight to the possible requirement for other hormones than FSH and LH.

FSH and LH are both glycoprotein hormones and heterodimers consisting of an alpha subunit, GPα which they have in common; and a beta subunit, which differs between the two hormones. DNA sequences encoding the putative FSH and LH subunits from *A. Anguilla* are known. All three subunits are relatively small; GPα contains 117 aa (amino acid) residues, while FSHβ contains 127 aa residues, and LHβ contains 140 aa residues (including signal peptides).

While glycosylation of glycoproteins are usually only important for serum half-life, and thereby indirectly for potency, FSH and LH are unusual by being totally dependent on glycosylation for function, in analysed species. All three subunits are glycosylated, but only N-linked glycosylation at GPα $Asn_{80}$ are essential for function. Fig. 4.4.1 shows the aa sequence of the FSH subunits from *A. anguilla*. Putative glycosylation sites are indicated.

Heterologous productions of glycoproteins are usually performed using either: yeast, insect cells, or cells from mammals. Glycans from yeast and insect cells differ substantially from glycans from vertebrates like mammals and fish. To avoid potentially negative effects of aberrant glycosylations, mammalian cells have been used for hormone production in this project.

The work is divided into i) production of antibodies towards FSH subunits ii) transient expression in CHO (Chinese hamster ovary) cells, and iii) production in stably transfected CHO cells. All through

the project genetic construction has been made for production of all three subunits (GPα, FSHβ and LHβ), but priorities have been on production of FSH subunits (GPα and FSHβ).


**Fig. 4.4.1.** Aa sequence of subunits in FSH. Signal peptides (green) are removed in the mature hormone. Glycosylation sites are indicated with red arrowheads. The essential glycosylation site is indicated with a double arrowhead.

**i) Production of antibodies towards FSH subunits**

Antisera towards GPα and FSHβ were developed in collaboration with Biogenes (Berlin, Germany) and Bam Biotech Co (Xiamen, China). Antigenic regions were selected from the sequence of GPα and FSHβ, avoiding glycosylation sites and cysteines, and corresponding peptides were synthesized. For GPα, two peptides were produced, one stretching 14 aa (Biogenes) and one stretching 20 aa (Bam Biotech). A single FSHβ peptide of 16 aa was produced (Biogenes). Two rabbits were immunized with each peptide, and antisera were collected.

Dotblotting experiments revealed that antisera from all six rabbits efficiently recognized the immunizing peptide (not shown). Dotblotting experiments were also used to identify the rabbit, of the two immunized with each peptide, that responded best to the peptide, and these three antisera were used for identification of heterologous FSH produced in expression experiments.

**ii) Transient expression in CHO cells**

Synthetic genes for GPα, FSHβ and LHβ, optimized for CHO cell expression, were designed and produced. Analysis of the native *A. anguilla* GPα, FSHβ and LHβ signal peptides (using SignalP 3.0), suggested that signal peptides would function efficiently in mammalian cells, and native signal peptides were thus preserved.

The synthetic GPα gene was inserted into the expression vector pcDNA3.1/Zeo (Invitrogen) thereby generating pUP938. FSHβ and LHβ genes were inserted into pcDNA3.1 (Invitrogen), generating pUP936 and pUP937.

pUP938 and pUP936 were used alone, and in combination, in transient transfection experiments using polyethylene (PEI) as transfection reagent in CHO DG44 cells. Cells and culture supernatants were harvested after 2, 4 and 8 days, and culture supernatants were analysed by SDS-PAGE and western blotting, se Fig 4.4.2.

pUP938 and pUP936 were also used alone, and in combination, in transient transfection experiments using the Freestyle system (Invitrogen), in CHO FreeStyle cells, for transfection. Cells and culture supernatants were harvested after 2, 4 and 6 days, and culture supernatants were analysed by SDS-PAGE and western blotting, se Fig 4.4.2.

Western blotting experiments showed that antisera obtained in i) were of varying quality. Antiserum towards GPα, produced in collaboration with Bam Biotech, efficiently recognized a protein from salmon pituitary extracts (presumably GPα) and a protein which only was present in culture supernatants from cells transfected with pUP938 (presumably GPα), se Fig 4.4.2. In contrast, antiserum towards GPα, produced in collaboration with Biogenes did not recognize proteins in neither salmon pituitary extracts nor pUP938 culture supernatants (not shown). Antiserum towards FSHβ, produced in collaboration with Biogenes, recognized proteins in salmon pituitary extracts, but did not recognize proteins in culture supernatants from cells transfected with pUP936, presumably because expression levels were low combined with a relatively low efficiency for this antiserum (not shown).

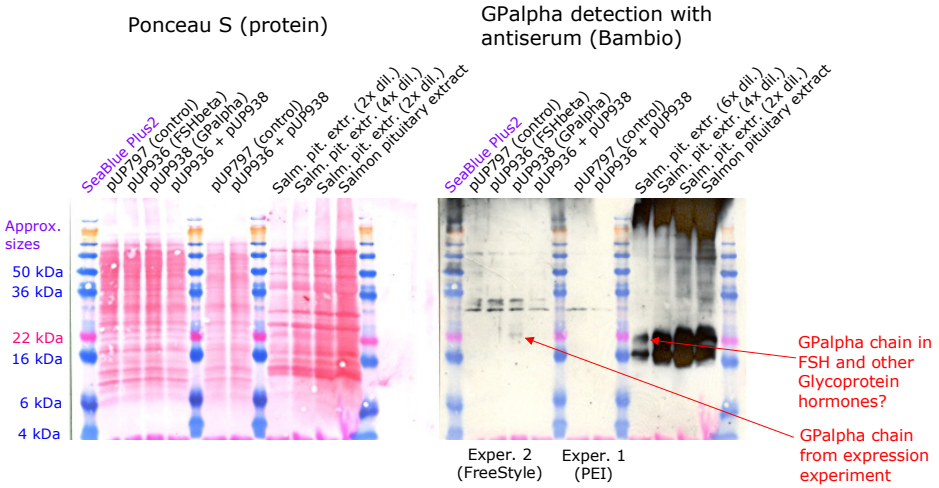


**Figure 4.4.2.** Western blotting experiment using anti-GPα. Culture supernatants from experiments using either PEI or Freestyle transfection reagents were loaded onto SDS-PAGE gels. Dilutions of a salmon pituitary are loaded as positive controls. Gels were subsequently blotted to nitrocellulose membranes and protein transfer was confirmed using Ponceau S stain (left panel). After removal of Ponceau S, membranes were probed with anti-GPα as primary antibody and anti-rabbit antibodies as secondary antibodies. Intense bands shows recognition of Salmon GPα from pituitary extract. A weaker band shows presence of GPα with FreeStyle transfection with pUP938. Higher molecular weight proteins are also recognized in all culture lanes. These are assumed to be unspecific recognition, since bands are also present with pUP797 and pUP936, neither of which contains heterologous GPα genes.

### iii) Production in stably transfected CHO cells

While transient transfection enables fast production of small amounts of protein, construction of stably transfected cells will typically result in higher protein titers, enable simple culture up scaling, and provide a better basis for further improvement of protein production. The CHEF1 system from CMC Biologics was used in combination with CHO DG44 cells for generation of stably transfected cells. In brief, new synthetic genes for GPα, FSHβ and LHβ were designed and inserted in the expression vector pNEF38. In order to create single constructions which enables production of both subunits of FSH and LH, reading frames for both subunits were inserted downstream of the EF-1α promoter of pNEF38. Translation of the downstream reading frame was enabled by insertion of an optimized ECMV IRES (encephalomyocarditis virus internal ribosomal entry site) sequence between the two reading frames. To increase the probability of successful expression of both reading frames, FSH constructions were made with either subunit gene in the upstream

position. In the genetic construction pUP969 the sequence of genetic elements is 5'-FSHβ-IRES-GPα-3' and in the construction pUP976 the sequence is 5'-GPα-IRES-FSHβ-3'.

Expression plasmids were used to transfect CHO DG44 cells (FreeStyle transfection) and stable transfected cells were selected over a period of 15 days. Culture supernatants were harvested and analysed by SDS-PAGE and western blotting using antisera produced in i). Fig.4.4.3 shows the result of an analysis.

Intense bands show recognition of GPα from both Salmon pituitary extract, and from cells transfected with pUP976. No bands are present with pUP969 indicating lower GPα expression from the ECMV IRES compared to when GPα is in the IRES upstream position (pUP976). A lower general protein yield is, however, seen in this lane (coomassie stained gel), and this might also explain the lack of GPα detection in this culture. The low protein yield was not investigated further.

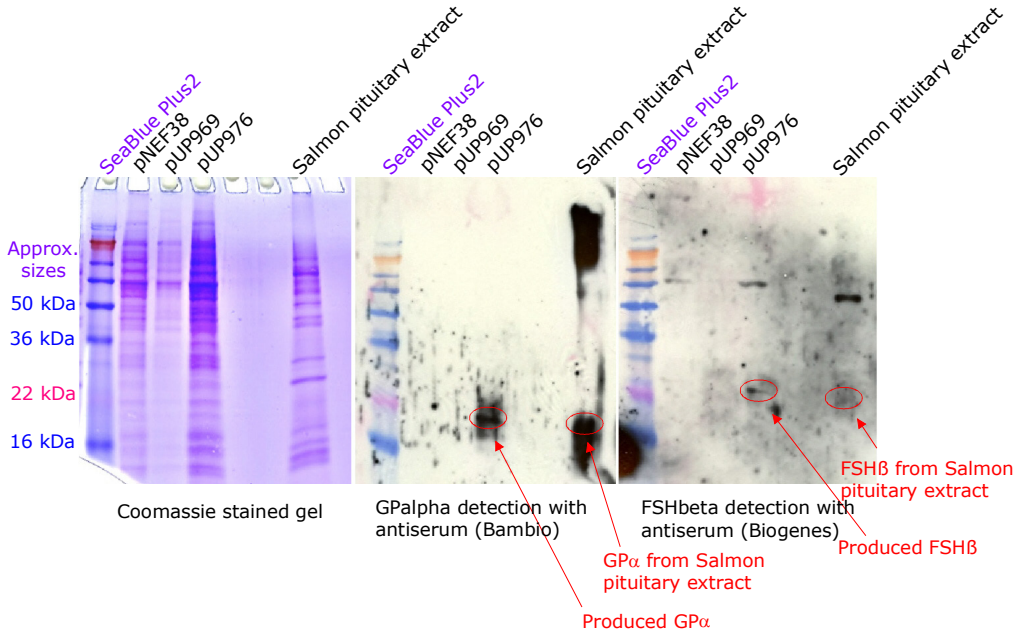


**Figure 4.4.3.** SDS page and western blotting of culture supernatants from stably transfected cells. Cells were transfected with pNEF38 (negative control), pUP969 and pUP976. Left pane shows a coomassie stained SDS-PAGE gel. In the middle pane an identical gel was blotted onto a nitrocellulose membrane and probed with anti-FSHβ. In the right pane another membrane is probed with anti-GPα.

On the anti-FSHβ probed blot, bands in pituitary extract are much weaker, and this possible results from both low amounts of FSH in these extracts (this is expected), and from a less efficient antiserum. Despite of the assumed lower FSHβ sensitivity, a band of the proper size is present in pUP976 supernatant, indicating FSHβ production. A band larger than 50 kDa is also present in this lane, but presence of the same band in pNEF38 supernatants shows that this is unspecific recognition of an unknown protein

The western blotting experiments showed that yields of GPα and FSHβ was strongly increased with stably transfected cells compared to when expressed by transient transfection.

**Conclusion**

We have successfully produced antisera which can be used to evaluate heterologous FSH and LH production. These antisera can also be used in the analysis of existing hormone products and in immunological studies on expression *in vivo* in eels and other fishes.

We demonstrated that detectable levels of the GPα subunit could be produced by transient transfection in mammalian cells. FSHβ production was also attempted, but could not be demonstrated. This was probably due to less efficient antibodies, compared to the GPα antibodies. Finally, we produced both GPα and FSHβ by stable transfected cells. The presence of both subunits was demonstrated by western blotting, and it is likely that these subunits are present in the form of complete FSH. Further analysis of structure, and *in vitro* and *in vivo* activity studies, would reveal whether this is the case. Stable FSH expressing cells is an advantageous basis for a future hormone production.

During this study, genetic expression constructions have been med for both FSH and LH production. Since the production of FSH is considered most important for the development of a new hormone preparation, this hormone was given priority through the work. LH production can however, be established in a relatively short time-frame from constructed expression plasmids.

### 4.4.3 Evaluation of ZF-implants as alternative maturing hormones

**Introduction**

The Zanter project aimed at testing the possibility of using implanted hormone producing cells as a source to mature female eels. Involved in this project was the University of Leiden, ZF- Screens (a Dutch commercial company), DTU Aqua and Bioneer. The rationale for using cell-implants is that the eels will be stimulated by FSH and LH in a more uniform and less stressing manner. It was expected that the use of implants would result in a better egg quality, a higher fertilisation percentage and an improved buoyancy of eggs during embryonic development.

**Evaluation of ZF-implants**

Experiments were planned and initially the ability of these cells to survive in eel tissue was tested. To evaluate the viability of implanted cells to survive in eel tissue indicator cells were injected at specific locations in the tissues (see Fig. 4.4.4).

The injected indicator cells contained lacZ - a gene that codes for β–betagalactosidase. This intracellular enzyme cleaves the disaccharide lactose into glucose and galactose. Cells that produce β–betagalactosidase will turn blue by X-gal staining, thereby making them detectable in the tissue and hence indicating whether the cell is alive or not.

**Approach**

In total, 16 silvering females were used in this test including 12 females with LacZ, 4 females with medium alone and 4 females with no injection at all (control). Females were sampled at week 1, 2,

3 and 4 after injection of the indicator cells using the following procedure. After terminating the fish using an over dosage of anaesthetics, the injection sites were located and a tissue sample (0, 5 $cm^3$) was removed using a scalpel. The block of tissue was then placed in Eppendorf tubes containing PBS. When the tissue was stained, PBS was removed, X-gal added, and the sample left at room temperature for 30 minutes. The tissue was then washed twice with buffer. Staining took place in total darkness overnight. Staining of the tissue was then visible to the naked eye.

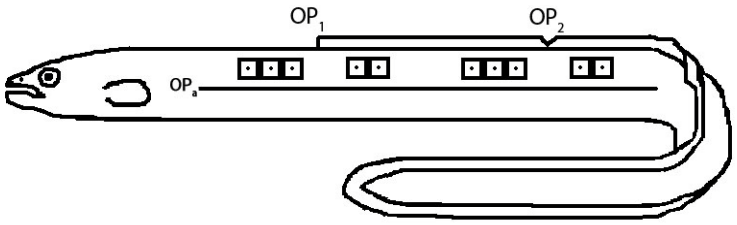


**Figure 4.4.4.** The squares depict the injection patches each with a size of 0.5cm x 0.5cm. Dots within the squares are needle insertions. $OP_1$: Orientation point dorsal fin; $OP_2$: Orientation point fin clips; OPa: Orientation point lateral line.

The degree of staining decreased steadily over time for the LacZ treated fish (Figure 4.4.5). A clear indication of blue colouring was observed in week one after injection, while only a reduced marking appeared in week two. In week three an additional staining was made to obtain a total staining period for 48 hours, however, only small blue dots were observed there. As a consequence, we altered the procedure for week four by taking two fish out of the aquarium; one was sliced in minor cubes and one cut out as a whole piece but handled in a larger container. No activity of the injected cells was obtained in any of these tests. Fish with medium did not respond to the staining at all. The females in two treated groups did not behave differently to the control and no mortality occurred.

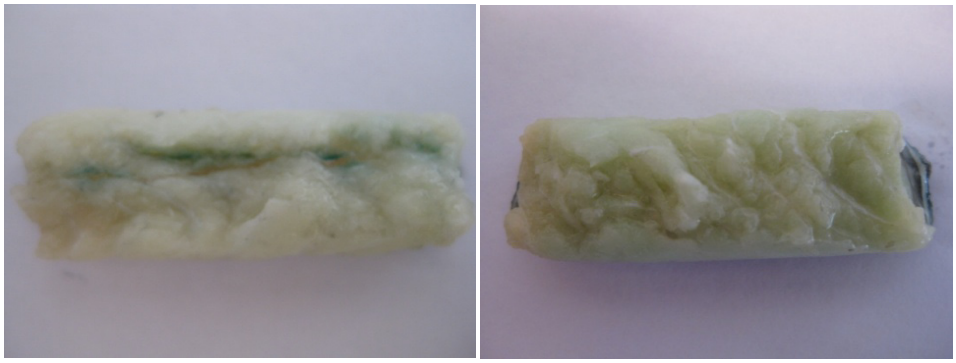

**Figure 4.4.5.** Left: Clear blue staining one week post injection of indicator cells. Right: No blue staining one week post injection of medium cells only.

**Conclusion**

The use of indicator cells proved unsuccessful. No cells survived even at high dosage when tested on eels in culture. The technique needs further development and it was concluded that at the present state further testing would be futile.

A state of development of the hormone producing implants allowing further tests was not reached by Leiden University and ZF-Screens during the project period. The planned collaborative experiments were cancelled and REEL project resources allocated to improvement of existing methods and hormone production using recombinant gene technology.

## 4.5 Task 5: Optimization of methods for fertilisation, incubation of eggs and hatching of larvae

*Sune Riis Sørensen, Jonna Tomkiewicz, Peter Lauesen, Christian Graver, and Tanja Kofoed*

### 4.5.1 Introduction

In the fertilisation experiments, egg and semen quality was evaluated and the right dosage of sperm to egg in fertilisation procedures was assessed. In addition, methods for incubating fertilized eggs and hatching of larvae were evaluated. The task of optimizing and standardizing the development and survival of embryos by improving methods was handled by BA, DEFA and DTU Aqua.

**Milestones**

**M 5.1:** Methods to estimate fertilisation success and survival rates as well as to evaluate egg and embryo quality were developed and implemented. (Section 4.5.1).

**M 5.2:** Standard protocols for sperm storage medium fertilisation and incubation were developed and implemented (Section 4.5.2).

### 4.5.2 Estimation of fertilisation success and survival rates, and to evaluate egg and embryonic quality

Maturation in European eel involves complex hormonal mechanisms and induced maturation using salmon pituitary extract (SPE) and DHP or DOC for final maturation and ovulation often results in low egg quality and fertilisation success. This hampers the production of high quality eggs, high fertilisation and survival rates during the embryonic phase and thereby mass hatching of viable larvae. Sperm production is well controlled and quality seems high and stable (Pérez et al. 2000; Tomkiewicz et al., 2011), however fertilisation capacity may vary. In order to enhance methods and procedures, evaluation criteria were developed and introduced at different steps. A system to grade quality of stripped, fertilised eggs was developed to provide an objective description of eggs harvested in a standardized way. This evaluation of egg quality is combined with quantification of egg production, standardised sperm application and assessment of fertilisation success. This evaluation is included in an egg monitoring program that is used to compare methods tested in relation to e.g. female hormonal treatment, fertilisation methods including sperm storage media and activation salinity.

### Egg monitoring

**Figure 4.5.1.** Monitoring procedure for accurate estimation of number of eggs incubated and fertilisation rate and subsequent mortality during incubation.

A method was developed to evaluate fertilisation rate of eggs 3-4 hours after incubation in saltwater. A random subsample of floating eggs and a mixed fraction was taken. The samples were photographed and image analysis used to categorise the eggs according to a set of criteria identifying different egg types. The classification system included five primary egg categories and additional sub-categories, which in combination were used in the evaluation of egg quality. Criteria are based on e.g. appearance and buoyancy characteristics (floating/sinking). This leads to separation of fertilised and non-fertilised eggs as well as quality within categories e.g. regularity of cleavages (Sørensen et al., *in prep*. b). In addition to the evaluation of egg quality and fertilisation success early after activation, an additional sample was taken prior to activation. This sample was used to evaluate the number of eggs incubated.

In order to estimate mortality during the incubation period, a procedure was developed where standardised sampling is performed during development in an incubator. Sampling is carried out at the onset of incubation and right before expected time of hatch, which enables an accurate estimation of mortality during this period. Figure 4.5.1 illustrates sampling using this procedure. Sampled eggs are counted and used to estimate total number of incubated eggs.

The first steps in the development of this monitoring protocol were made during the ROE III project. The method was further developed and tested during the REEL experimental series. The egg monitoring was implemented on 45 egg batches (30 out of 62 stripped in series REEL A, 7 out of 19 in second series B, and 8 out of 11 stripped in series C). The method has proven to be an efficient tool to evaluate the reproduction success in relation to diet, treatment and fertilisation protocols.

## 4.5.3 Standard protocols for sperm dilution, fertilisation and incubation

For comparison of the egg and larval production success obtained using different treatments and methods, it is valuable that the fertilisation procedures are carried out in a standardized way. This reduces noise in the results and is valuable also for small scale experiments. The fertilisation procedures developed include a standardized way to strip the females and males, evaluate sperm quality, dilute semen, mix gametes in standardised ratios, and activate gametes. The procedures thus standardize the sperm to egg ratio and uses a medium to ensure an even distribution of sperm and eggs when mixed. A medium for sperm dilution resembling natural seminal plasma of European eel (Asturiano et al. 2003 and 2004) was tested as an extender of the dilution medium. These pioneering tests using diluted semen and standardised ratios in fertilisation procedures were successful, and protocols formulated taking into account the individual variation of sperm quality and quantity produced by males. The general quality assessment of male semen involves estimation of sperm motility and density.

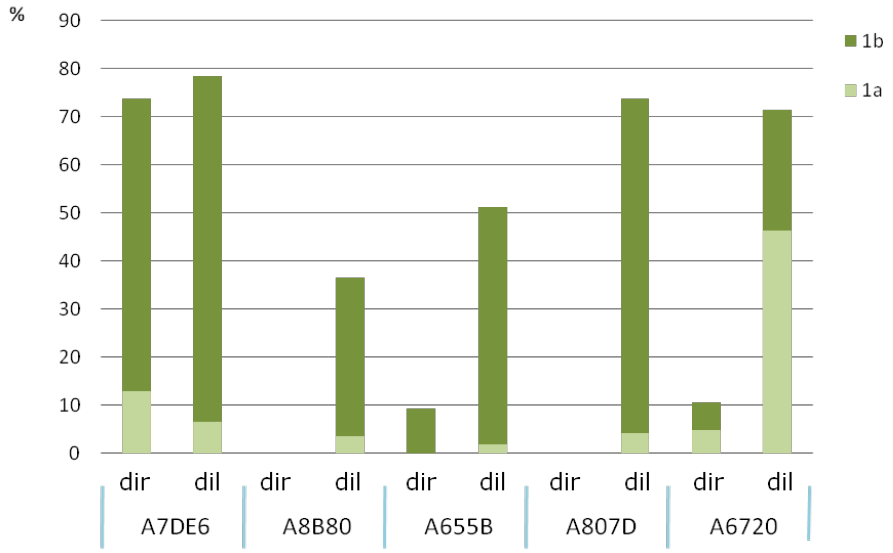


**Figure 4.5.2.** Quality of fertilized eggs applying fresh semen stripped directly over the eggs = *dir* and diluted semen in a standardized concentration = *dil* for five females identified by pittag numbers (A7DE6, A8B80, etc.). The dark green bars represent the percentage of type 1b eggs, i.e. fertilized eggs showing irregular cell cleavage. The light green bars represent type 1a eggs characterized as fertilized eggs with symmetrical and regular cell cleavages. The Y-axis shows percent of eggs with the 1a or 1b type of cells out of fraction of floating eggs assessed 4-5 hours post fertilisation (HPF). Data originates from Series A.

An experiment was conducted to compare the two types of fertilisation procedures using 1) fresh sperm from males stripped directly over the eggs and 2) sperm from the same males, diluted in the medium using standardised procedures and applied in a standardised sperm to egg ratio. The egg monitoring method was used to evaluate fertilisation success. Egg batches from 8 females were fertilised using semen from 3-4 males either directly stripped over the eggs or diluted to a standardised density. Semen from the same males was used in the tests for all egg batches. The fertilisation procedures were conducted in parallel to enable comparison of treatments. Three hours after fertilisation, egg monitoring was conducted to estimate fertilisation success and egg quality.

The fertilisation rate was higher using the standardised fertilisation procedure than when applying fresh sperm stripped over the eggs and even in two cases resulted in fertilised eggs where none were observed using undiluted fresh semen. In addition, the proportion of fertilised eggs with regular cleavage (type 1a) compared to irregular (type 1b) tended to be higher, when using diluted semen and standardised egg:sperm ratio. Figure 4.5.2 illustrates the results obtained from 5 females out of the 8 tested egg batches. The egg batches stripped from the remaining 3 females were of poor quality and fertilisation was not observed. The percentage of type 1a eggs was higher in 4 out of the 5 cases when using the dilution. The method has two advantages, firstly the ratio of sperm to egg is kept constant, and secondly the dilution of semen facilitates mixing of sperm and eggs. Based on these preliminary results, a protocol for harvesting semen and preparing standardised sperm dilutions was developed. The method is easy to apply and facilitates the fertilisation procedure as the semen dilution can be prepared in advance (Sørensen et al., *in prep.* a).

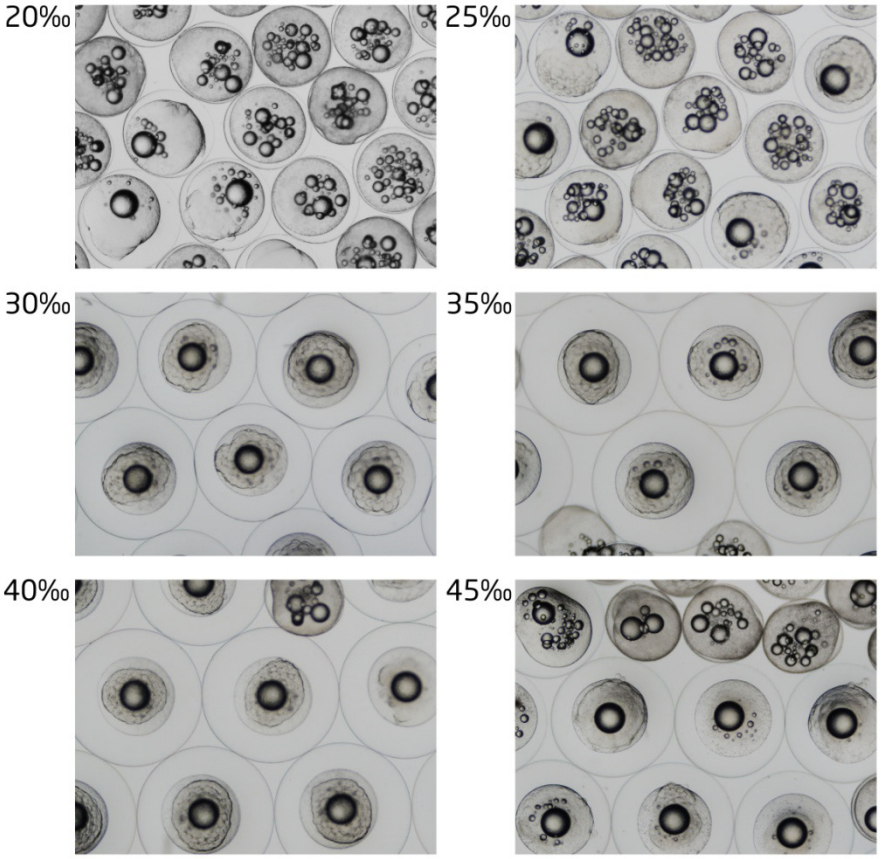


**Figure 4.5.3.** Pictures of eggs 4 hours post fertilization (hpf) activated using different salinities in the activation water. Salinities indicated, states the ambient salinity during fertilization. All eggs illustrated originated from same female.

### 4.5.4 Optimized activation salinity

After harvesting and mixing of gametes, the gametes are activated by adding saltwater. The salinity in the activation water as well as temperature and the time lapse between harvest and activation may affect fertilisation success and development characteristics. Experiments using different salinities in the activation water showed that the optimal range of ambient salinities during fertilisation were in the range 30-40 ‰ (Fig. 4.5.3). An effect is seen both in the swelling of the egg and the development of the blastula.

In relation to these experiments also the effect of activation, salinity and egg size on the buoyancy of the eggs was investigated using a density gradient column. The results indicate that the salinity concentration in the activation process affects buoyancy through differences in the development of the perivitelline room and egg density (Sørensen et al., *in prep.* c).

### 4.5.5 Time between harvest and activation

The time lapse between harvest and activation of gametes is another factor important for *in vitro* fertilisation success of eel eggs (Otah et al. 1996). Fertilisation rates decrease with time from harvest to the subsequent activation of gametes and an attempt was made to assess the rate of deterioration. An experiment was conducted to test fertilisation capacity in relation to time after harvest of eggs using standardised procedures and activation at six points in time from 3 minutes

up to 2 hours 20 minutes post harvest. Standard egg monitoring was conducted, but in the evaluation, the eggs were categorised into fertilised and unfertilised eggs without sub-division.
The results of the experiment are illustrated in Figure 4.5.4. A high fertilisation rate was observed during the first interval of 7 minutes, while a significant reduction in fertilisation rate was observed during the following interval i.e. from 7 to app. 40 minutes. This confirmed Japanese observations suggesting that the time lapse until activation should be kept at a minimum in the improved standard procedures.

In this preliminary study, eggs and sperm were mixed after harvesting using the dilution medium that contains e.g. *Bovine Serum Albumine* (BSA) and left until activation. Further tests are planned to invest if the composition of the medium affects the results.

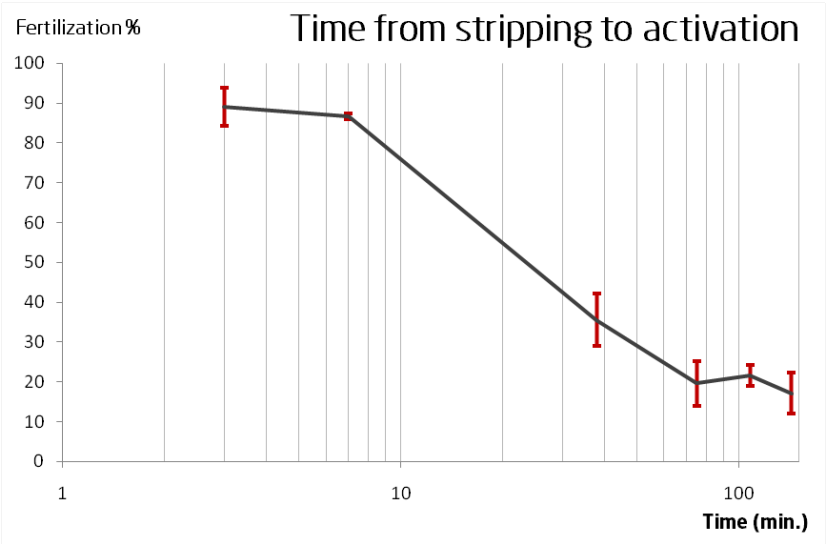


**Figure 4.5.4.** Relationship between percent fertilized eggs and time from harvesting (stripping) to activation of mixed eggs and sperm cells. The fertilization percent is plotted at each time point ±SD. Data originates from experiments in series C.

## 4.6 Task 6: Development of larvae tanks and establishment of larvae cultures

*Peter Lauesen, Peter Munk, Sune Riis Sørensen, Svend Steenfeldt, Bjarne Vestbö Niels Hjermitslev, and Jonna Tomkiewicz*

### 4.6.1 Introduction

Eel larvae are little developed at hatch, and they develop through the yolk-sac stage over app. 12 days. At this time their mouth parts are fully developed and ready to feed. The eel larvae seem sensitive to turbulence, air bubbles and light, and depending on larval stage, their movements are either directed towards the bottom (as yolk-sac larvae) or towards the surface when the yolk is exhausted (leptocephalus larvae). These circumstances were considered during the development of new types of rearing tanks. For feeding larvae, BioMar developed an inert larval feed in collaboration with DTU Aqua. DTU Aqua and BA collaborated on the development of larval tanks.

**Milestones**

**M 6.1:** Larval feed developed and tested. (Section 4.6.2).

**M 6.2:** Larval tanks developed and tested. (Section 4.6.3).

**M 6.3:** Protocols for culturing larvae and methods to determine survival and growth were developed. (Section 4.6.4).

## 4.6.2 Larval rearing

During the experimental period in REEL C a combined high egg production, fertilisation rate and egg development success resulted in mass hatch of larvae. The test facility did not need that large amounts of larvae for the planned experiments and transport of viable eggs and larvae to other locations was tested.

Transport to the aquaculture unit of DTU Aqua in Hirtshals was successfully conducted. The aquaculture unit is fully equipped to handle large batches (>100.000) of eggs and larvae. Two batches originating from females in group M in REEL C were transported to Hirtshals. App. 15 hours prior to hatch, eggs were selected and gently transferred to 40 l seawater filled plastic containers with an estimated amount of 50.000 to 80.000 eggs in each container. Plastic containers were then carefully placed in polystyrene boxes.

The egg bags were transferred to Hirtshals by car and upon arrival, eggs were placed in incubators until hatching. Temperature during transport was kept at the same level as at the test facility (19.5 to 20.5 °C). Seawater in the containers held a salinity of 36-38 ppt. After hatch, the larvae were transferred to a fully recirculated water system and kept at a temperature of approx. 20 $^{o}$C. Salinity varied slightly according to the buoyancy of the larvae and was in the range of 36 – 40 ppt.

At day 12 feeding experiments were initiated using a slurry diet formulated by Niels Hjermitslev, BioMar and Jonna Tomkiewicz, DTU Aqua, and produced by Bjarne Vestbö, BioMar A/S. The larvae in these trials did not show systematic feeding behaviour. Longevity of larvae in the two batches was:

Batch 1: number of larvae hatched app. 100.000, larval longevity 15 days.
Batch 2: number of larvae hatched app. 200.000 larval longevity 20 days

Eggs from the same batches were similarly transported to DTU Aqua in Charlottenlund for specific experiments on behaviour. Traditional larval tanks were applied for the rearing.

## 4.6.3 Larval culture facilities

As the maturation protocols became more successful and embryonic survival enhanced the subsequent larval production, the demand for suitable containers for larval culture increased. In particular the last series of the REEL project, series C, provided good opportunities for testing larval rearing systems as high numbers of good quality larvae were available. Main challenges include to reduce larval contact with the bottom and sides of the containers and to ensure a stable water quality.

### Larvae culture tanks

Several pilot studies were performed in order to build a system, where water enters from below and creates a laminar upwards flow in the container, keeping the larvae in the water body. This water flow in the system minimizes the risk of larval contact with the bottom and debris in the culture tanks.

A prototype system was made including 4 larval containers (Figure 4.6.1). The system consists of two interconnected reservoirs holding 250 l's of water each and separate pumps that pump water 2 meters up into two water towers above the larval tanks. The water tower systems are supplied with a series of valves of push in type of one way valves enabling outflow only when PE tubes are attached in these (seen in Figure 4.6.2).

During the REEL project series C, the system was tested with promising results. Importantly, larvae were kept suspended during incubation. The majority of larvae in the water body remained buoyant and the water quality was maintained high without the use of antibiotics needed in static cultures.

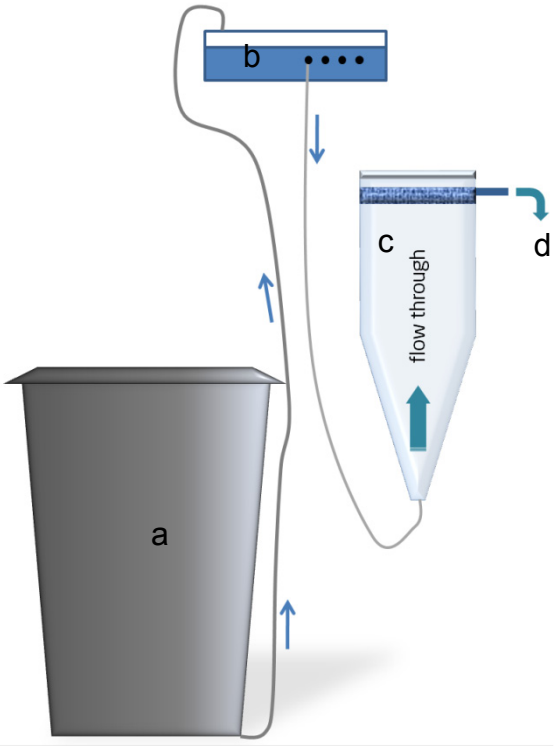


**Figure 4.6.1.** The larvae culture tank in principle. a: reservoir (system containing two), b: water tower, c: larvae tank (made in 4 replicates), d: outflow.

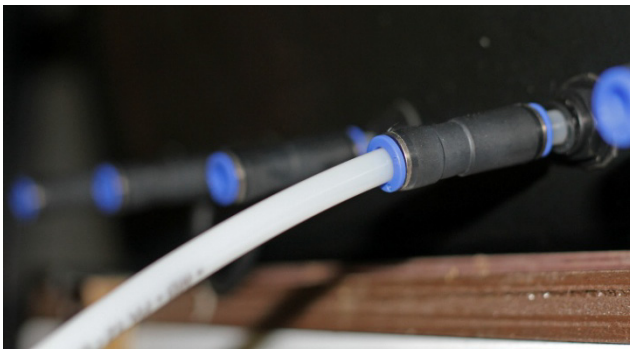

**Figure 4.6.2.** Push-in hose connection for attaching each larval tank. Valves are closed automatically when detaching the tube.

A mesh was placed above the inlet at the bottom of the tank, sealing it and creating a wider area of water inflow thereby avoiding that larvae come in contact with the inlet. Even though the flow was weak, the current was sufficient to disturb and damage larvae close to the inlet.

## 4.6.4 Protocols for larvae cultures

The opportunity to test rearing protocols and larval development increased with the increased availability of larvae towards the end of the project.

Both larval abundance and quality varied much between production series. As described above, a new promising rearing system was developed and knowhow in culture of yolk-sac larvae was obtained. However, irrespective of apparently adequate rearing conditions, the vast majority of larvae died around day 6 post hatch. Only very few larvae survived until the time where they are fully developed and expected to feed i.e. app. 12 days post hatch at 20 °C.

A protocol for larval sampling was used to map larval development. Larval growth and development during the yolk-sac stage was documented through systematically obtained photographs of larvae, and subsequent image analysis. A range of morphological characteristics were defined and standard measures were carried out routinely. Important measures include length to follow larval growth and relative size of the yolk-sac and oil droplet. The measures of relative size of the yolk-sac showed significant differences between batches of larvae as well as between larvae from different series/treatments. These differences also influenced survival characteristics with a higher mortality of larvae with relatively small yolk-sacs (Fig. 4.6.3).

Larval abundance was roughly estimated on a daily basis at different locations of larval rearing. Survival of larvae from the same female was observed to be fairly similar, irrespective of the location and rearing facilities (DTU Aqua Lyksvad, Hirtshals and Charlottenlund). This included similar characteristics in e.g. periods of high mortality.

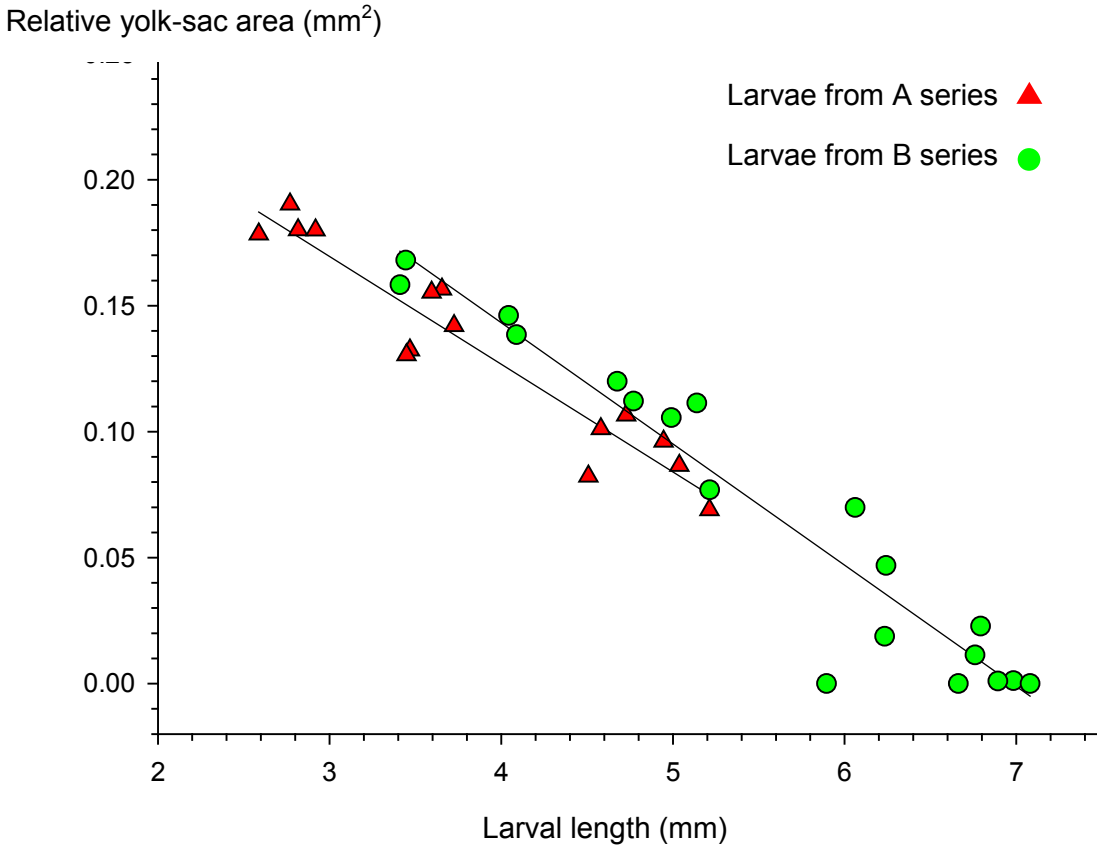


**Figure 4.6.3.** Relative yolk-sac area and corresponding standard length (SL) of larvae in two experimental series of REEL i.e. series A, red triangles, and series B, green circles. A trend line is fitted through date from each series.

## 4.7 Task 7: Operation and technical development of research facility

*Lars Tybjerg, Peter Lauesen, Thomas Hornum, and Jonna Tomkiewicz*

### 4.7.1 Introduction

At the experimental facility, automatic control systems (PLC) and temperature regulation of tanks were installed to upgrade the system and allow different temperature regimes under broodstock maturation. The intention was to rear broodstock females with implants at a higher temperature as the hormone producing cells thrive at slightly higher temperatures than at the standard temperature used. Also laboratory conditions were improved and security controlled. This task is primarily managed by BA and DTU Aqua.

#### Milestones

**M 7.1:** PLC system installed, fume hood established and security conditions authorized.

### 4.7.2 Technical development

#### PLC – Installation and functionality

The ability to monitor and control water temperature regimes in fish aquaculture systems is useful in all standardised experiments. At the research facility monitoring of temperature regimes using handheld measuring instruments was replaced by modern PLC surveillance (Fig. 4.7.1). In addition, temperature control of different broodstock rearing systems was established (in the range 15°C - 25°C) allowing different temperature regimes in different systems.

The installation of the PLC system temperature control and data logging was carried out in two tempi by project partner Billund Aquaculture Service (BA).

**Figure 4.7.1.** PLC system for registration and control of physical-chemical conditions in the recirculation systems

*Step 1: Logging temperature*

A PLC system including temperature sensors and data logging was prepared and tested. Sensors were installed in broodstock containers, incubation and larval rearing room. This included sensors in each of the broodstock systems and registration of ambient room temperature. In the incubation room sensors were installed in incubators and registration of ambient room temperature. A sensor was installed to measure ambient temperature in the larval room.

Logging of data from sensors was programmed to occur at fixed time intervals. Threshold values for activation of the alarm system at low and high temperatures were defined. The system was tested and adjusted including fine tuning and calibration of sensors. The technical challenges

were solved in collaboration with the supplier and after some weeks the system functioned as intended. For future development of the PLC, the system was prepared to log salinity levels in the water basins. Data are regularly analysed for evaluation and documentation.

An alarm on the PLC control panel sends an SMS to the person in charge if temperatures out of range are registered.

A comparison between measurements of the temperature logging of the PLC and the handheld equipment was made (Table 4.7.1). Three water systems were selected for evaluation and the difference in measured temperatures is given below:

**Table 4.7.1.** Calibration of PLC. Comparison of temperature measurements in 3 recirculation systems measured using different equipment.

| Equipment | Temperature ($^{o}$ C) | | |
|---|---|---|---|
| | **System 1** | **System 2** | **System 4** |
| COND3110 (handheld) | 19.2 | 19.6 | 19.8 |
| OXYGUARD (handheld) | 19.2 | 19.6 | 19.8 |
| PLC | 19.3 | 19.8 | 20.5 |
| Relative deviation | 0.1 | 0.2 | 0.7 |

A comparison of the measurements from the handheld equipment and the PLC showed a difference in measurements in the range of 0 to 0.7 °C. All measurements showed a trend towards higher temperature from System 1 to 4. However, measurements of handheld equipments showed no difference within systems, the PLC values deviated from the handheld indicating a need for calibration and systematic control.

Within systems, the measurements of handheld equipments showed no difference in separate parts of the system. The results were thus unaffected by whether the measurements were taken in either one of the tanks containing the eels, the small reservoir or the tank containing the pump.

*Step 2: Control of water temperature*

In the second step of the PLC installation, equipment was installed to allow cooling of water in two of the systems and heating of water in another two systems. Preliminary tests demonstrated the ability to cool the water to 15$^{o}$C with the temperature regulation being PLC controlled and easy to handle. In order to perform experiments at a higher water temperature, PLC connected heating elements were mounted in another two systems. Tests demonstrated that temperatures at 25$^{o}$C could easily be obtained and maintained.

**Light regimes**

Day and night light regimes play an important role in the hormonal control of fish reproductive physiology and shifts in light intensity may cause stress in larvae. The PLC system was

programmed to display specific day/night light cycles including twilight periods. Modifications were made to control rooms independently. Also the strip light was changed to “natural light” imitating natural daylight. The diurnal cycles can be programmed or adjusted to follow specific patterns.

### Enhancement of laboratory facilities

The laboratory facilities of the experimental facility were enhanced to improve safety in the working environment. A fume hood and a ventilated cabinet for storage of chemicals were installed (Fig. 4.7.2). In general, the facility was upgraded to be in compliance with Danish legislation and fulfil requirements to work with chemicals, hormones etc. applied in the daily work.

**Figure 4.7.2.** Fume hood and cupboard for chemicals installed at the laboratory at the research facility.

## 5 Conclusion

### Coupling to previous research and future studies

During the past decade, researchers working on reproduction of eels made significant progress in Japan as well as in Europe. Outstanding results for the Japanese eel were obtained in 2010, where the life cycle was closed and an F2 generation produced. New results for European eel were obtained in a number of Danish projects, where larvae hatched in large numbers completing the yolk-sac stage and leptocephali were produced for the first time in history.

The Danish projects were led by DTU Aqua in collaboration with the aquaculture industry represented by Danish Eel Farmers Association (DÅP), Billund Aquaculture Service (BA), Copenhagen University, Faculty of Life Science (KU-Life) and the fish feed producer BioMar. The projects “Artificial Production of Eel II and III (ROE II and ROE III)” were carried out in the period 2005-2008. The projects ROE II and III received by the Ministry of Food, Agriculture and Fisheries and the European Commission through the Financial Instrument for Fisheries Guidance (FIFG) respectively the Danish Food Research Program 2006.

The results were a break-through for the artificial reproduction of the European eel. For the first time mass hatching of larvae was achieved several times, and new records for the survival of the

larvae were set each year: 5 days in 2006, 12 days in 2007 and 18 days in 2008. Researchers in the project ROE lll successfully hatched larvae which within a period of app. 12 days went through the yolk-sac stage and the first leptocephali of European eel were produced. From these larvae a single batch survived to enter a first feeding test.

The present project REEL accomplished through three series of experiments to consolidate previous results and extended the longevity of larvae to 20 days post hatch. Maturation potential and methods to induce maturation were further tested using different treatments and new diets to enhance female broodstock were developed for application. In particular, fertilisation procedures and monitoring techniques were enhanced, and incubation and larval culture systems were substantially improved. In addition to culture conditions, development of larval rearing tanks and feed for larvae have been developed. Also the DTU Aqua experimental facility was improved by enhanced experimental and laboratory facilities.

There are still many tasks to be resolved for a future production of larvae and glass eels, but through the ROE and REEL projects, useful knowledge was obtained about a range of factors, some constraints were combated, and new methods developed and tested. Comparisons between farmed eels and wild eels show good results for farmed eels e.g. less disease, being accustomed to handling etc., but also constraints e.g. their nutritional stage that differs from the wild eels. Therefore, new feeds for the mother fish is being developed in order to improve the quality of the broodstock, and expectedly also of the eggs and larvae. By comparing farmed broodstock and wild silver eels in reproduction experiments valuable knowledge about body and egg composition is obtained.

The applied method to artificially mature the eels with regular injections of hormones does not reflect the natural maturation cycle, which constrains severely both the capacity for reproduction, the embryonic development and the survival of larvae. The present project therefore included development of new maturation methods e.g. changed dosage of hormone. Methods for production of eel hormones using recombinant gene technology were successfully developed and may replace attempts to develop hormone producing cells that may prove difficult to regulate.

The consolidating and development of methods in the REEL project provided the basis for the establishment of an EU collaborative research project: *Reproduction of European Eel: Towards a Self-sustained Aquaculture* (PRO-EEL) coordinated by DTU Aqua. REEL included the partners DTU Aqua, KU-Life, Danish Eel Farmers Association, Billund Aquaculture Service, BioMar, Bioneer and of which four are integrated in the PRO-EEL project that in total has 15 international partners.

# 6 Dissemination

**2011**

Tomkiewicz, J., Kofoed, T. M.N., and Pedersen, J.S. 2011. Assessment of testes development during induced spermatogenesis in European eel (*Anguilla anguilla*). Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science, 3: 106-11.

Støttrup, J.G., Jacobsen, C., Tomkiewicz, J., and Jarlbæk. H. 2012. Modification of essential fatty acid composition in broodstock of cultured European eel, *Anguilla anguilla* L. Aquaculture Nutrition (*Accepted*).

**2012 - 2013**

Støttrup, J.G., Jacobsen, C., Tomkiewicz and Peter Lauesen. Fatty acid composition in cultured and wild female broodstock and eggs of European eel *Anguilla anguilla*. (*In prep)*.

Sørensen S.R., Gallego, V., Pérez, L., Asturiano, J.F, and Tomkiewicz, J. Standardised sperm application for *in vitro* fertilization of European eel (*Anguilla anguilla*). (*In prep.* a*)*.

Sørensen, S.R., Lauesen, P., Munk, P., and Tomkiewicz, J. Quality criteria and biophysical characteristics of fertilized eggs from European eel (*Anguilla anguilla*) matured in captivity. (*In prep.* b*)*.

Sørensen, S.R., Munk, P., Lauesen, P., Tomkiewicz, J. Effects of activation salinity on fertilisation rate and egg development in European eel (*Anguilla anguilla*.) matured in captivity. (*In prep.* c).

Tomkiewicz, J., Munk, P., Sørensen, S.R., Støttrup, J.G., Lauesen, P., Graver, C., Jacobsen, J. and Jarlbæk, H. Hormonal induction of maturation and production of larvae of European eel (*Anguilla anguilla*). (*In prep.)*

# 7 References

Asturiano J.F., Pérez L., Marco-Jiménez F., Olivares L., Vicente J.S. and Jover M. 2003. Media and methods for the cryopreservation of European eel (*Anguilla anguilla*) sperm, *Fish. Physiol. Biochem.,* 28, 501–502.

Asturiano JF, Pérez L, Garzón DL, Marco-Jiménez F, Penaranda DS, Vicente JS, and Jover M, 2004. Physio-chemical characteristics of seminal plasma and development of media and methods for the cryopreservation of European eel sperm. *Fish. Physiol. Biochem*., 30: 283–293.

Otah H. Kagawa H., Tanaka H. Okuzawa K. and Hirose K. 1996. Changes in fertilisation and hatching rates with time after ovulation induced by 17, 20P-dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica*. *Aquaculture* 139: 291-301.

Pérez L, Asturiano JF, Tomas A, Zegrari S, Barrera R, Espinos JF, Navarro JC, and Jover M. 2000. Induction of maturation and spermiation in the male European eel: assessment of sperm quality throughout treatment. *J. Fish. Biol.,* 57: 1488–1504.

Tomkiewicz, J. and Jarlbæk, H. 2008. Artificial Reproduction of Eels: ROE II og II B. *DTU Aqua report no.,* 180-08 (*in Danish*).

Tomkiewicz, J. 2008. Status report: Artificial Reproduction of Eels, ROE III. Report for the Ministry of Food, Agriculture and Fisheries (*in Danish*).

Tomkiewicz, J. and Sørensen, S.R. 2008. Final report: Artificial Reproduction of Eels III – culture and larvae, ROE III-LC. Report for the Ministry of Food, Agriculture and Fisheries (*in Danish*).

DTU Aqua
National Institute of Aquatic Resources
Technical University of Denmark

Jægersborg Allé 1
2920 Charlottenlund
Denmark

Tel: + 45 35 88 33 00
aqua@aqua.dtu.dk
www.aqua.dtu.dk